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Preservative Potential of Thai Herb Extracts Combined with Bacteriocin from *Bacillus velezensis* BUU004 for Controlling Food Spoilage and Pathogenic Bacteria in Dried Crushed Seasoned Squid

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ABSTRACT

This study aimed to develop a novel biopreservative to replace chemical preservatives currently used in Thai dried seafood products. A mixed extract of chili spur pepper and lemongrass was evaluated for its preservative potential in dried crushed seasoned squid. The mixed extract (80 mg/ml) showed weak activity against bacteria, lowering the total viable count (TVC) measured in dried squid during a 28-day storage at room temperature; although, at 160 and 320 mg/ml, the applications of mixed extract demonstrated a similarly strong inhibitory efficacy to each other, but higher than that of the 80-mg/ml mixed extract. However, some spoilage from *Bacillus* species and fungi were found in the treated samples. Then, a novel preservation technology was developed by incorporating the mixed herb extract (160 mg/ml) with a semi-purified preparation of bacteriocin from *Bacillus velezensis* BUU004 (SPP-BV). A significant (p<0.05) decrease in TVC was observed in the herb-SPP-BV-treated squid, along with the absence of fungal and staphylococci growth during a 28-day refrigerated storage. The novel combination was also investigated for its inhibitory activity against the foodborne pathogen, *Bacillus cereus*, in dried squid using the MPN method with results showing a substantial reduction in *B. cereus* numbers during a 28-day refrigerated storage. The mode of action of this novel combination was pathogen cell lysis due to ruptured cell walls, observed by SEM. Finally, administration of the novel preservative cocktail had no degenerative effects on the sensory qualities (color and odor) of the squid. These results suggest that the mixed herb extract combined with SPP-BV has promising potential as an alternative food preservative for

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controlling food spoilage and pathogenic bacterial growth, and improving Thai dried seafood product quality.

Keywords: *Bacillus cereus*; *Bacillus velezensis*; Bacteriocin; Dried seafood; Herb extract

1. Introduction

Traditional dried seafood products are one of the most popular cuisines in Thailand, with a production of 51,953.70 tons in 2017, equivalent to approximately $160 million in revenue [1]. Various dried seafood products in Thailand struggle with harmful damage from microbiological and chemical changes that seem to be key factors affecting their safety and quality. During the past decade, several reports have pointed out that dried seafood products distributed in eastern provinces of Thailand (ca. 36-52%) contained a total viable count (TVC) over the limit allowed by Thai regulatory standards [2-5]. In addition, *Bacillus cereus*, a food-borne pathogen, is the dominant species in a variety of traditional dried seafood products, e.g., salted fish, dried mussels, seasoned fish strips, seasoned squid, and seasoned crab [5]. The pathogen poses a health hazard by causing gastrointestinal diseases such as diarrhea and emesis, by producing the enterotoxins haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE), cytotoxin K (CytK), and an emesis-inducing toxin cereulide [6]. This health hazard has led to the research and development of strategies to reduce serious risk of foodborne illness.

In recent years, there has been a dramatic increase in the demand for minimally processed food products that contain low levels, or are free of, synthetic preservatives and chemical additives due to the suspicion that they can cause intoxications, allergies, cancers, and other degenerative diseases [7]. This trend is also reflected by a European Union Directive [8] aiming to decrease the use of chemically derived food preservatives, particularly nitrites. This has led to the dire need for discovering effective, safe, and food compatible technologies that protect food products from pathogen contamination, and that respond to growing consumer demand for products that fit a healthy lifestyle. Nowadays, naturally occurring compounds, e.g., bacteriocins from beneficial bacteria and plant-derived extracts, have gained the interest of food manufacturers and food safety researchers, as a biopreservative that works without resulting in organoleptic deterioration [9].

Herbs and spices have been used as food additives for millennia; besides being used to improve taste, their use as food preservatives is well-established due to their lack of negative side effects, economic benefits and a long history of safe use [10]. In Thailand, several types of herbs and spices attract the attention of health-conscious consumers and are valued for their antimicrobial activity, not just their flavor and fragrance. The antimicrobial properties of herb and spice extracts can be used for a wide range of applications in various industries including pharmaceuticals, cosmetics, and food processing. Thai herb extracts and essential oils, e.g., ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citratus*), cinnamon (*Cinnamomum cassia*), garlic (*Allium sativum*), clove (*Eugenia caryophyllus*), chili (*Capsicum annuum*), kaffir lime (*Citrus hystrix*), cumin (*Cuminum cyminum* L.), shallot (*Allium ascalonicum* L.) and galangal (*Alpinia galangal*) contain a variety of bioactive components that contribute to their antibacterial activity against food-borne pathogens [11-12]. However, a high concentration of certain herb extracts/essential oils is required to attain the same effect in foods as is seen in culture medium experiments, which is thought to be a key limitation for their application in the food industry [13]. High concentrations of herb extracts/essential oils can have degenerative effects on taste, flavor, and odor leading to an undesirable sensory experience of the food product. As such, a combination of herb extracts with antimicrobial peptides produced by beneficial bacteria at optimal concentration may be able to overcome this limitation by minimizing concentrations and decreasing organoleptic impact, while still ensuring the safety of the food [14]. Using such a combination of these compounds could become a desirable alternative to synthetic preservatives, offering healthier food products to consumers and providing economic benefits to producers.

Bacteriocin, a bacterial ribosomally synthesized peptide with antimicrobial activity, has been recognized as a safe food biopreservative and has received increasing interest as a means to extend shelf-life and enhance food safety [9]. In recent studies, a semi-purified preparation containing bacteriocin from *Bacillus velezensis* BUU004 (SPP-BV) had a strong inhibitory effect on foodborne Gram-positive and Gram-negative bacteria both *in vitro* and in a dried seafood model [4, 15]. Therefore, the aims of this study were to evaluate the antibacterial activity of a mixture of lemongrass and chili spur pepper (*Capsicum frutescens* Linn) extracts and assess the preservative potential of the mixed herb extract used in conjunction with SPP-BV for controlling growth of spoilage bacteria and the pathogenic *B. cereus* in dried crushed seasoned squid throughout storage. Sensory evaluation of the dried seasoned squid applied with the mixture of herb extract and the SPP-BV was also performed.

2. Materials and Methods

2.1 Herb extraction

Lemongrass (stalk), and chili spur pepper (fruit) were collected from a local botanical garden in Chon Buri Province. The plants were washed using running tap water, cut into small pieces using a sterile table knife, and then dried in a plant drier at 35°C for 72 h. After being ground using an electric blender, the dried plant materials were separately added into a flask containing 95% ethanol used as an extractant at a ratio of 1:10, material to extractant, and agitated in a shaking incubator (New Brunswick Innova 434, Edison, New Jersey, USA) at 120 rpm, 30°C for 72 h [16]. After vacuum-filtration using a Whatman filter membrane No.1, the supernatant was evaporated at 40°C and 175 mbar using a rotary evaporator (R-205, Buchi, Flawil, Switzerland). The ethanolic extract was stored in an amber bottle at -20°C until use.

2.2 Efficacy of mixed chili spur pepper and lemongrass extracts for controlling TVC in dried seasoned squid

In a preliminary study, ethanolic extracts of ginger, black peppercorn, lemongrass, kaffir lime and chili spur pepper at 5 to 80 mg/ml were individually evaluated for their *in vitro* antibacterial activity against food-spoilage and the foodborne pathogens *Edwardsiella tarda*, *Pseudomonas aeruginosa*, as well as the methicillin-resistant *Staphylococcus aureus*, isolated from dried seafood products in our laboratory. None of the extracts exhibited satisfactory activity, but a mixed herbal extract of lemongrass and chili spur pepper showed a strong inhibitory potential against the harmful bacteria (data not shown). Consequently, the mixture of chili spur pepper and lemongrass extracts was used in this study for controlling TVC in dried squid following a protocol described by Butkhot et al. [4]. The herb extract combination was produced by mixing chili spur pepper and lemongrass extracts at a ratio of 1:1, then adjusted to attain concentrations of 80, 160, and 320 mg/ml. A 2×2 cm piece of dried crushed seasoned squid purchased from a retail store in Chon Buri, Thailand was made using sterile scissors. Then, a 4 cm^2 piece of squid sample was introduced with a small volume (0.1 ml) of either sterile distilled water (control) or the mixed herb extract at the abovementioned concentrations. The squid samples were kept in a sterile zip-lock bag at room temperature and withdrawn at 15 min, 2, 4, 7, 14, 21, and 28 days of storage for TVC measurement.

2.3 Efficacy of mixed chili spur pepper and lemongrass extracts combined with SPP-BV for controlling TVC and *B. cereus* in dried seasoned squid

Despite the mixed herb extract being capable of decreasing TVC and eliminating spoilage staphylococci in dried squid samples, fungal and *Bacillus* growth were observed during storage. This indicates that a higher efficient biopreservative is required. Our recent study revealed that bacteriocin from *B. velezensis* BUU004 had preservative potential in dried seafood products due to its inhibitory action against several foodborne pathogens [15]. In order to improve the biopreservative performance, a combination of the mixed herb extract (160 mg/ml) with the SPP-BV, and storage at 2-4 ºC was assayed in this experiment.

B. velezensis BUU004, an aquaculture probiotic isolated from pond sediment of black tiger shrimp (*Penaeus monodon*), was sub-cultured three-time onto Trypticase Soy Agar (TSA; BD Difco, Sparks, MD, USA) and then inoculated into Trypticase Soy Broth (TSB; BD Difco). After incubation at 30°C, 200 rpm for 18 h, a cell supernatant containing bacteriocin was harvested using a centrifuge (5804 R, Eppendorf, Hamburg, Germany) at 8,000*g*, 4 °C for 10 min, and then passed through a 0.45-µm membrane filter (Sartorius, Gottingen, Germany). The SPP-BV was produced by adding ammonium sulfate at 80% saturation into the obtained cell-free supernatant and then stirring gently at 4°C overnight [4, 15]. The precipitate was harvested by centrifugation at 10,000*g*, 4°C for 30 min and re-suspended in 50 mM sodium phosphate buffer (pH 7.0). The obtained protein suspension was dialyzed against a dialysis tube (cutoff 1 kDa, Spectra/Por 7, Spectrum Laboratory, Los Angeles, CA, USA) at 4°C overnight and then passed through a 0.45 µm membrane filter. The filter-sterilized SPP-BV was assessed for bacteriocin activity in arbitrary unit (AU) following a method described by Butkhot et al. [4, 15]. The SPP-BV used in this study contained 800 AU/ml of bacteriocin activity.

2.3.1 Effect on TVC

The squid samples were prepared as aforementioned. A square sample was introduced with one of these supplements: 1) sterile distilled water (control), 2) SPP-BV (800 AU/ml), 3) mixed chili spur pepper and lemongrass extracts at 160 mg/ml, and 4) mixed chili spur pepper and lemongrass extracts at 160 mg/ml plus SPP-BV at 800 AU/ml. Owing to the presence of spoilage *Bacillus* and fungus in the dried squid samples treated with the mixed herb extract at room temperature during storage in this study, we employed hurdle technology by integration of low temperature and preservative addition for controlling spoilage bacterial growth. After air-drying in a biosafety cabinet for 15 minutes, all samples were stored at 2-4°C in a refrigerator. During the 28-day storage, the squid were sampled at 15 min, 1, 7, 14, 21, and 28 days for TVC evaluation.

2.3.2 Effect on pathogenic B. cereus

Apart from food-spoilage bacteria, inhibitory activity of the mixed herb extract in combination with SPP-BV against the food-borne pathogenic *B. cereus* was investigated. Cell suspension was prepared by growing *B. cereus* TISTR687 in TSB overnight at 30°C then adjusting concentration to approximately 10^4 CFU/ml. The suspension (0.5 ml) was slowly dropped onto whole surface of a 2 × 2 cm squid sample using an autopipette. Following air-drying in a biosafety cabinet for 15 min to allow maximum adhesion to the food matrix [4], the samples with pathogen inoculation were divided into 4 batches and introduced with 0.1-ml tested additives: 1) sterile distilled water (control), 2) the SPP-BV (800 AU/ml), 3) mixed chili spur pepper and lemongrass extracts at 160 mg/ml, and 4) mixed chili spur pepper and lemongrass extracts at 160 mg/ml plus SPP-BV at 800 AU/ml. The samples were allowed to air-dry for 15 min. Afterwards, the squid sample of each batch was separately kept in a sterile plastic zip-lock bag at 2-4 °C. *B. cereus* count on the dried squid samples was enumerated at 15-min, 1, 7, 14, 21 and 28 d post-inoculation.

2.4 Bacteriological analysis

Total viable bacteria were counted following a method recommended by the US FDA [17] with some modifications. A portion (0.5 g) of the sample from each trial at defined intervals was homogenized with 0.1% (w/v) peptone water (4.5 ml) in a sterile stomacher bag. A serial 10-fold dilution was made in 9 ml of peptone water. An aliquot (0.1 ml) of each dilution was spread-plated onto Plate Count Agar (BD Difco). After incubation at 35±2ºC for 24 h, all colonies were counted as TVC and expressed as colony forming units (CFU) per g of sample. All experiments were performed in triplicate. Isolated bacteria grown on the medium were identified on the basis of Gram staining, cell morphology, and biochemical characteristics following a classical method [18] and API test kits (bioMerieux, Marcy I' Etoile, France).

Enumeration of *B. cereus* was conducted using a three-tube most probable number (MPN) method modified from the US FDA [19]. Briefly, the dried squid sample (2 g) was diluted with Butterfield's phosphate buffered dilution water, and then three successive 10-fold dilutions were made. An aliquot of each dilution (1 mL) was added into a set of three tubes of TSB plus 0.15% Polymyxin B and incubated at 30±1°C for 48±2 h. Tubes with turbidity were seeded onto Mannitol Egg Yolk Polymyxin agar prior to incubation at 30±1 °C for 18-24 h. Typical colonies of *B. cereus* (pink colony with lecithinase enzyme activity observed by the presence of an opaque zone around a colony) were streaked onto Nutrient Agar for biochemical and morphological tests recommended by the US FDA [19]. The MPN tables were used to calculate the approximate number of bacteria per gram.

2.5 Scanning electron microscopic observation

To assess bactericidal activity, logarithmic phase *B. cereus* TISTR687 suspension (1×10^8 CFU/ml) in TSB was introduced with tested additives: 1) sterile TSB (control), 2) SPP-BV (800 AU/ml), 3) mixed chili spur pepper and lemongrass extracts at 160 mg/ml, and 4) mixed chili spur pepper and lemongrass extracts at 160 mg/ml plus SPP-BV at 800 AU/ml, and incubated at 30±1°C for 20 h. The untreated and treated *B. cereus* suspensions were centrifuged (8,000*g*, 4 °C for 10 min), washed two-times, then re-suspended in phosphate-buffered saline (pH 7.2). The collected cells were prepared for electron microscopic assay as detailed in Butkhot et al. [4]. Bactericidal activity of the tested additives was evaluated by observing growth of the untreated and treated cells using a spread-plate technique on TSA plates.

2.6 Sensory evaluation

Sensory evaluation was conducted in two sample groups: the control and the most active combination between the mixed chili spur pepper and lemongrass extracts (160 mg/ml) in conjunction with SPP-BV (800 AU/ml) in dried crushed seasoned squid. A panel of five individuals evaluated odor and color of the samples following the standard sensory evaluation protocol [20]. Prior to sensory analysis, dried squid samples were each assigned a unique, random 4-digit code, then served on separate plates. The trained panelists scored the smell and color of the squid using a three-point hedonic scale: 3 = excellent (normal appearance of color, very good odor), 2 = satisfactory (appearance of fairly changed color, fairly good odor) and 1= unsatisfactory (appearance of unusual color, off-odor e.g., rancidity and/or staleness). Sensory evaluations were performed on the first (1 d) and last (28 d) days of refrigerated storage.

2.7 Statistical 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 at a significance level of $p<0.05$. Tukey's test was applied to find any difference of parameters found in the ANOVA test. All statistical analyses were performed using Minitab Version 18.1.0.

3. Results

3.1 Efficacy of mixed chili spur pepper and lemongrass extracts for controlling TVC in dried seasoned squid

The mixture of chili spur pepper and lemongrass extracts (80 mg/ml) was introduced to the dried crushed seasoned squid. During a 28-day storage at room temperature, TVC in the dried seasoned squid remained relatively constant, within the range of 10^4–10^5 CFU/g. A significant difference ($p<0.05$) in TVC was measured between the control and mixed-herb-extract treated groups, except at day 2, 21, and 28 of storage (Fig. 1).

To increase inhibitory efficacy, mixed herb extract at either 160 or 320 mg/ml was added to the dried crushed seasoned squid prior to storage at room temperature. A significantly ($p<0.05$) lower TVC of the dried seasoned squid treated with the mixed herb extract at 160 or 320 mg/ml was observed, compared to that of the control (Fig. 2). Additionally, the mixed herb extract, at 160 and 320 mg/ml, was capable of eliminating predominant spoilage bacteria, e.g., *Staphylococcus fleurettii*, but some spore-forming bacilli remained prevalent, including *B. firmus*, *B. alcalophilus*, *B. insolitus*, *B. stearothermophilus*, and *B. pasteurii* (data not shown). Fungus also grew on the culture medium obtained from the squid sample exposed to the mixed herb extract (320 mg/ml) and untreated sample at day 28 of storage (Fig. 2). As a consequence, the mixed herb extract at 160 mg/ml was selected to be used in the subsequent experiment.

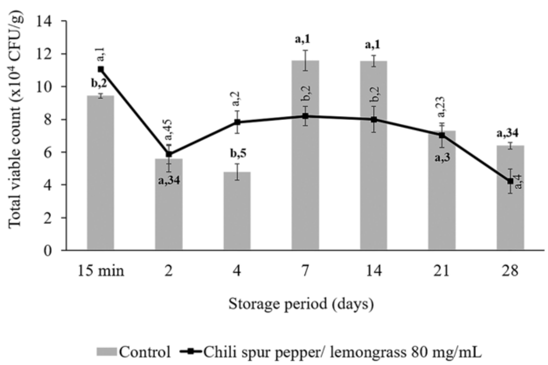

Fig. 1. Change in TVC in dried crushed seasoned squid supplemented with mixed chili spur pepper and lemongrass extract at 80 mg/ml during 28 days of storage at room temperature. Superscript letters denote significant difference ($p<0.05$) between the control and treated groups. Superscript numbers denote significant difference ($p<0.05$) over storage period.

3.2 Efficacy of mixed chili spur pepper and lemongrass extracts combined with the SPP-BV for controlling TVC in dried seasoned squid

During a refrigerated storage for 28 days, TVC in squid samples treated with the mixed herb extract in conjunction with SPP-BV were significantly ($p<0.05$) lower than those of the control and other treated squid, except at 15-min, day 1,

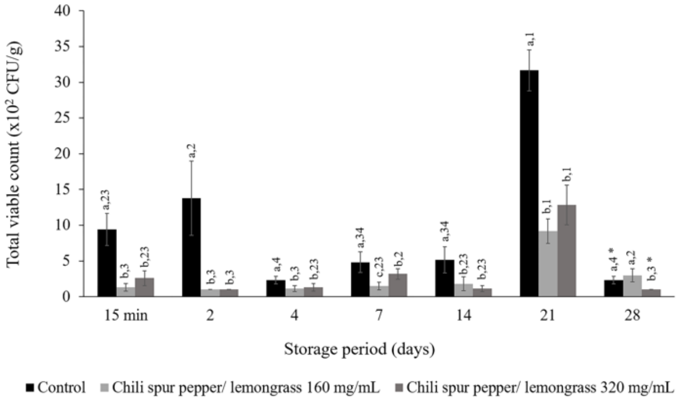

Fig. 2. Total viable count (TVC) in dried crushed seasoned squid supplemented with mixed chili spur pepper and lemongrass extracts at 160 mg/ml, 320 mg/ml, and distilled water (control) during 28 days of storage at room temperature. Superscript letters denote significant difference ($p<0.05$) among treatments. Superscript numbers denote significant difference ($p<0.05$) over storage period. * = presence of fungal growth.

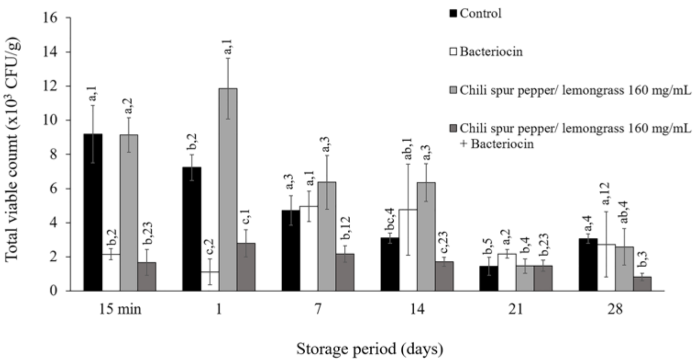

Fig. 3. Total viable count (TVC) in dried crushed seasoned squid supplemented with the SPP-BV (800 AU/ml), the mixed chili spur pepper and lemongrass extract at 160 mg/ml, mixed chili spur pepper and lemongrass extract at 160 mg/ml in combination with the SPP-BV (800 AU/ml), and distilled water (control) during 28 days of refrigerated storage. Superscript letters denote significant difference ($p<0.05$) among treatments. Superscript numbers denote significant difference ($p<0.05$) over storage period.

Table 1. Antibacterial effect on *B. cereus* TISTR687 of the SPP-BV (800 AU/ml), mixed chili spur pepper and lemongrass extract (160 mg/ml), and mixed chili spur pepper and lemongrass extract in combination with the SPP-BV during 28 days of refrigerated storage.

Treatments	Sampling time (days post-inoculation; MPN/g)					
	15 min	1	7	14	21	28
Distilled water (control)	>1,100	460	240	150	93	43
SPP-BV	93	43	28	43	21	14
Mixed chili pepper and lemongrass extracts	43	38	36	28	16	9.2
Combination of chili pepper and lemongrass extracts and SPP-BV	43	36	29	23	15	9.2

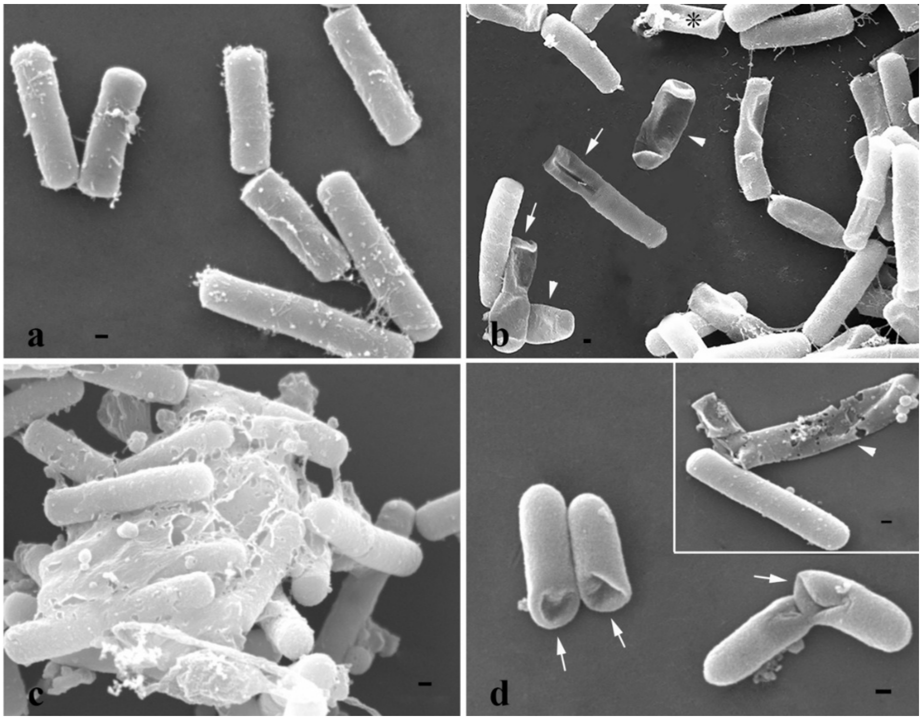

Fig. 4. Microscopic images of *B. cereus* treated with or without the additives for 20 h. a) normal structural cells of untreated *B. cereus*, b) cell shrinkage (asterisk), pore formation (arrow), and ghost cells with efflux of cytoplasmic materials (arrowhead) of the SPP-BV-exposed *B. cereus*, c) agglutination and adhesion of *B. cereus* treated with mixed chili spur pepper and lemongrass extracts, and d) pore formation (arrow) of *B. cereus* treated with the mixed herb extract plus the SPP-BV with inset showing collapsed, flat and empty cell (arrowhead). Bar = 200 nm.

and day 21 of storage (Fig. 3). TVC decreased significantly (p<0.05) to 1.67±0.76×10^3 CFU/g in squid exposed to the herb extract plus SPP-BV at 15 min post-introduction, and then was relatively constant in the range of 0.83±0.22×10^3 to 2.80±0.79×10^3 CFU/g throughout storage. There was no significant (p>0.05) difference between the SPP-BV-treated squid and the herb-extract-SPP-BV-treated squid during the first 24 h of storage. Thereafter, TVC in the SPP-BV-treated group was in the range of 2.18±0.26×10^3 to 4.95±0.89×10^3 CFU/g, significantly (p<0.05) higher than that of the herb-extract-SPP-BV-treated group (8.33±0.16×10^2 to 2.17±0.48 ×10^3 CFU/g). TVC in the mixed-herb-extract-treated groups were comparatively variable between 1.47±0.42 ×10^3 and 1.19±0.18×10^4 CFU/g and were significantly (p<0.05) different from the herb-extract-SPP-BV-treated squid. Neither *Staphylococci* (*S. saprophyticus* and *S. lentus*) nor fungal growth was observed in the squid samples introduced with the herb extract plus SPP-BV during refrigerated storage, but some *Bacillus* species were largely isolated including *B. licheniformis*, *B. mycoides*, *B. circulans*, and *B. pumilus* (data not shown).

3.3 Effect of mixed chili spur pepper and lemongrass extracts combined with the SPP-BV on *B. cereus* growth in dried seasoned squid

B. cereus number in the control was >1,100 MPN/g at 15 min post-inoculation and thereafter slowly declined during a refrigerated storage, until dropping to 43 MPN/g at 28 days of storage (Table 1). On the contrary, there was a moderate reduction in *B. cereus* (93 MPN/g) at 15 min post-storage and 14 MPN/g at the end of storage. *B. cereus* populations in the mixed-herb-extract, and the mixed herb extract plus SPP-BV groups reduced substantially from 43 MPN/g at 15 min post-inoculation to 9.2 MPN/g at the end of experiment.

3.4 Scanning electron microscopy

As depicted in Fig. 4a, cell surfaces of the control *B. cereus* were smooth and intact in appearance. On the other hand, severe changes to the membrane surfaces of *B. cereus* cells treated with SPP-BV were seen, e.g., collapsed structure, cell shrinkage, pore formation, and efflux of cytoplasmic materials (empty and flaccid cells; Fig 4b). Agglutination of most *B. cereus* cells treated with the mixed-herb-extract supplement was observed and adjacent cells were also adhesive (Fig 4c). Exposure to the mixed herb extract plus SPP-BV of *B. cereus* induced destruction due to pore formation (Fig 4d), collapsing, and leaving ghost cells with cytoplasmic material completely lost (Fig 4d inset). All of these changes in the three treated groups may cause cell mortality and reflect the possible mechanisms of bactericidal action due to the absence of growth of the 20-h treated *B. cereus* cells on the culture medium.

3.5 Sensory characteristics

The results obtained by sensory trained panelists revealed that there was no difference in sensory scores of dried crushed seasoned squid treated either with the mixed herb extract plus SPP-BV or distilled water (control) in terms of color (Fig. 5 and 6) and odor (Fig. 6) during a 28-day refrigerated storage. These results indicate compatibility of the mixed herb extract (160 mg/ml) and SPP-BV (800 AU/ml) in the dried seafood model in regards to sensory acceptability.

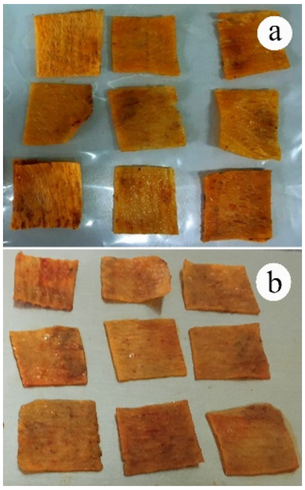

Fig. 5. Appearance of normal color of dried crushed seasoned squid introduced with (a) distilled water (control) and (b) mixed herb extract with SPP-BV.

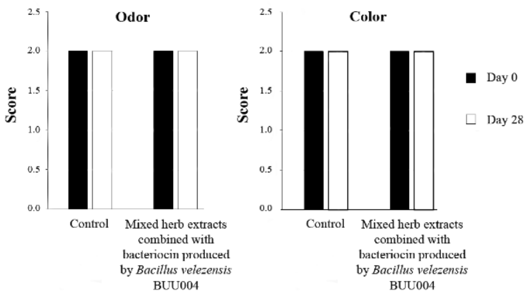

Fig. 6. Sensory scores of dried crushed seasoned squid added introduced with distilled water (control) and the mixed herb extract (160 mg/ml) combined with SPP-BV (800 AU/ml) during a 28-d refrigerated storage.

4. Discussion

A combination of multiple herb extracts produces a great inhibitory action on microbial growth without resulting in organoleptic impact on food products. In this study, administration of the mixed extract of lemongrass and chili spur pepper at 160 and 320 mg/ml resulted in a satisfactory reduction in TVC in dried squid products. Our results were consistent with several other studies focused on developing plant-extracts or essential-oil combinations to be used in food products. Kong et al. [21] employed a mixed herb extract (honeysuckle, *Scutellaria*, and *Forsythia suspense* Thunb) and mixed spice extract (cinnamon, rosemary, and clove oil) to control spoilage bacteria in vacuum-packaged pork chops and observed a 1.8-2.3-log reduction of TVC during a 28-day refrigerated storage. However, addition of both the mixed extracts caused a stronger discoloration and production of off-odors in the vacuum-packaged pork chops. This was in contrast to our results, in which the sensory scores remained unchanged. Similarly, a mixture of extracts from rosemary (*Rosmarinus officinalis*) and mint (*Mentha longifolia* L. Hudson) was able to suppress the growth of spoilage bacteria below the allowable limit, control the pH value, and prevent lipid oxidation in beef sausage during storage at 4°C [22]. The researchers also claimed that the shelf-life of the sausages containing the herb mixture was doubled, compared to the control [22]. In general, the shelf-life of fresh and processed products corresponds to the storage duration until spoilage. The point of spoilage can be defined by some criteria, e.g., threshold of the acceptable level of microbiological quality and chemical indicators as well as unacceptable off-color, off-odor and off-flavor. As a consequence, additional indicators recommended by food administration agencies, such as pH value and rancidity should be further studied to estimate the shelf-life of the dried squid samples.

In spite of our mixed herb extract being capable of reducing TVC and eliminating *Staphylococcus* contamination, *Bacillus* and fungal growths were found in the squid samples at the end of the storage period. This indicated a validation period and a limited inhibitory spectrum against food-spoilage microorganisms of the mixed extract in dried seafood products. Survival of spoilage contaminants in this study may involve, at least in part, their resistance to harmful stresses in dried seafood matrix and an insufficiently high concentration of herb extract [23]. Such phenomenon may also be associated with the herb extract’s volatility and biochemical reaction with food components, e.g., proteins, carbohydrates, and fats. Hayouni et al. [24] reported a negative impact on inhibitory efficacy due to binding and/or solubilizing ability of phenolic compounds (carvacrol and thymol) in proteins and fats, resulting in a reduction of their availability for bactericidal action in ground meat during storage. In addition, the mixed herb extract in the present study seemed to require a relatively long contact time against food spoilage bacteria, supported by the higher TVC seen in the squid samples treated with the mixed herb extract compared to the control, during the early phase of storage. It is accepted that inhibitory activity of plant extracts is dependent on concentrations and contact time intervals. The growth pattern seen is in agreement with that reported by Ramli et al. [25]. They revealed that the ability of *Syzygium polyanthum* L. leaf extract to eliminate/kill foodborne pathogens, e.g., *Listeria monocytogenes*, *P. aeruginosa*, *E. coli*, *S. aureus*, *Vibrio parahaemolyticus*, *V. cholerae*, *Klebsiella pneumoniae*, *Proteus mirabilis,* and *Salmonella Typhimurium*, concentrations held equal, increased with exposure duration. Further study focusing on contact time of the mixed herb extract against spoilage and pathogenic bacteria and stability of the mixed herb extract in dried seafood products should be done to expedite the processes involved in the deployment of these antimicrobial extract combinations in seafood products.

The inhibitory spectrum of herb extracts and essential oils is broadened enormously with the presence of bacteriocins, a finding supported by the significant reduction in TVC and *B. cereus* and the absence of staphylococci and fungal contaminants in dried seasoned squid introduced with the mixed herb extract together with SPP-BV observed in this study. These results are corroborated by another study by Field et al. [26]. A combination of bioengineered derivative nisin V with low concentrations of either carvacrol or trans-cinnamaldehyde demonstrated a potent effect by extending the lag phase by several hours and significantly reducing viable cells of *L. monocytogenes* in laboratory media, chocolate milk drink, and chicken noodle soup. The administration of nisin V and the essential oils carvacrol and cinnamaldehyde at low concentrations may play a key role in implementing the essential oils in food preservation without deteriorative organoleptic effects. Grande et al. [27] also investigated the efficacy of bacteriocin and enterocin AS-48, in combination with a variety of essential oils, e.g., carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*- coumaric acid, citral, and hydrocinnamic acid in various vegetable sauces. The inhibitory efficacy of the combined treatment was dependent greatly on the type of sauce and the phenolic compounds. The enterocin AS-48 (80 μg/ml) plus either 126 nM carvacrol or 20 mM hydrocinnamic acid served to completely inactivate *S. aureus* in carbonara sauce during a 30-day storage. The results indicated that chemical composition of food and the phenolic compounds may have a great influence on the activity of the combined additive. Inhibitory action against food spoilage and pathogenic bacteria of our combined additive would have significant implications in preventing gastrointestinal, diarrheal, and emetic syndromes caused by *B. cereus*, and enhancing dried seafood quality.

The active compounds present in the extracts from lemongrass and chili pepper have been identified in previous studies. Citral chemotype: geranial, neral, and limonene present in lemongrass extract/essential oil has a wide inhibitory spectrum against pathogenic *Escherichia coli* O157:H7, *Sal. Typhimurium*, *S. aureus,* and *L. monocytogenes* [28]. The major active components in chili pepper extract are cinnamic acid and *m*-coumaric acid, which contribute to exerting antibacterial action against foodborne pathogens [29]. In the present study, herb-extract-exposed *B. cereus* cells exhibited agglutination and cohesion with adjacent cells, as examined by SEM. Mortality of *B. cereus* in this study may also be attributed to other modes of action of herb extracts, such as attacking the cell membrane bilayer attack, destroying membrane integrity, increasing membrane permeability, and disturbing cellular activities [13]. Likewise, severe damage to the structural architecture of the SPP-BV-treated *B. cereus* cells was observed. The biochemical mechanisms of bacteriocins produced by *Bacillus* species correspond to typical destabilization of the cellular structure, destruction of membrane integrity, formation of transient pores, efflux of intracellular fluids, and ultimately cell mortality [30].

This study also clearly showed that the mixed lemongrass and chili spur pepper extract combined with SPP-BV possessed antibacterial action against pathogenic *B. cereus*. Its bactericidal activity may have been due to synergistic effects, and may be relevant to the alteration of cell structure, cell lysis, and consequently cell mortality. Ettayebi et al. [31] reported that growths of *L. monocytogenes* and *B. subtilis* were completely inhibited when thymol, a main active compound of thyme, in combination with nisin Z was applied. They explained that the inhibition might be associated with thymol-induced destabilization of the bacterial membrane structure through attacking the cytoplasmic membrane and changing the membrane permeability as a consequence of an increased concentration of intracellular nisin, a change in the proton motive force, an inhibition of enzymatic systems, leakage of a variety of molecules and ions, ultimately leading to cell death. Moreover, Pol and Smid [32] reported a reduction in viable counts of *L. monocytogenes* and *B. cereus* treated with combined carvacrol and nisin. Antibacterial activity of nisin might be potentiated through carvacrol function by prolonging the lifetime of pores created by nisin or increasing the number and size of the pores formed. Further study is needed to investigate the antibacterial mechanism of the combined additive, such as its effects on DNA and protein synthesis, and its interactions with food ingredients; such knowledge would help explain its synergy.

Sensory evaluation of dried seasoned squid was conducted with consideration of odor and color acceptability by the trained panelists. The results revealed that the addition of the mixed herb extract together with SPP-BV had no detrimental impact on the sensory qualities of the dried squid. Although the effect of a combination of herb extracts (lemongrass and chili spur pepper) with bacteriocins on sensory characteristics has never been reported on, negligible deterioration of organoleptic quality was conclusively demonstrated when plant-derived substances combined with bacteriocins were applied in meat systems. For example, Govaris et al. [14] reported that a combination of oregano essential oil (0.6 – 0.9%) with nisin at 500 – 1,000 IU/g showed strong antibacterial activity against *Sal. Enteritidis* while maintaining organoleptic acceptability of minced sheep meat during refrigerated storage.

5. Conclusion

A mixed herbal extract from lemongrass and chili spur pepper at 80 mg/ml showed a weak inhibitory effect on growth of spoilage bacteria in dried crushed seasoned squid. Addition of the mixed herb extract at 160 and 320 mg/ml resulted in significant reductions in TVC. The mixed extract (160 mg/ml) could not totally eliminate *Bacillus* contamination, while growths of several species of *Bacillus* and fungi were observed in samples treated with the higher concentration, at the end of experiment. Interestingly, a combination of the mixed herb extract with SPP-BV significantly reduced TVC and pathogenic *B. cereus* and eliminated staphylococci and fungal contaminants in dried squid samples during the 28-day refrigerated storage. SEM results confirmed the cell lysis of *B. cereus* caused by the combined additive. Apparent inhibition efficacy of the mixed herb extract combined with SPP-BV without a deteriorated sensory quality suggests that our combined additive has preservative potential for controlling the growth of contaminant and pathogenic bacteria, and enhancing the safety and quality of dried seafood products in Thailand. Application of multiple food biopreservatives in minute amounts may provide superior benefits to the application of a large amount of a single preservative, in terms of ensuring biosafety and maintaining the organoleptic quality of food products.

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