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Bacteriocin from *Bacillus velezensis* BUU004 as a Seafood Preservative: Antibacterial Potential, and Physical and Chemical Qualities of Dried, Seasoned, and Crushed Squids

Pornpimon Soodsawaeng[1], Nanticha Rattanamangkalanon[2], Traimat Boonthai[1], Verapong Vuthiphandchai[3], Subuntith Nimrat[2]*

[1]Biological Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand
[2]Department of Microbiology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand
[3]Department of Aquatic Science, Faculty of Science, Burapha University, Chon Buri 20131, Thailand
***Corresponding author e-mail: subunti@buu.ac.th; subunttbt@gmail.com**

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Abstract
Food safety of seafood-based products has become an important health threat in Thailand. Simultaneously, potential hazards posed by the use of chemical preservatives have prompted the advent of alternative technologies. Bacteria-derived substances have attracted interest as biopreservative to respond to health conscious demand of consumers. In order to enhance biosafety quality from a farm to fork cycle of traditional seafood products, bacteriological assessment during multiple steps related to preparation of dried, seasoned and crushed squids was conducted. Total viable counts (TVC) in the ranges of 10^2 - 10^3 CFU/g were observed across a series of the preparation from rinsing to 2nd sun-dry phases. Homemade seasoning sauce composed mainly of Thai spices and flavoring was the common source of spoilage bacteria supported by the highest TVC population and diversity. Three bacterial genera belonging to *Bacillus*, *Kocuria* and *Staphylococcus* existed predominantly in the prepared squids. In the subsequent phase of study, antibacterial potential, and mode of action of a semi-purified solution containing bacteriocin from *B. velezensis* BUU004 (SPS-BV) against pathogenic *B. cereus* were investigated. The SPS-BV (800 AU/mL) exhibited strong bactericidal activity towards *B. cereus* through cell lysis. Lastly, biopreservative potential of the SPS-BV was evaluated in the post-prepared dried squids during 28-day storage at room temperature. The SPS-BV was as effective as commercial nisin for controlling food spoilage bacteria along with significant reductions in moisture content and a_w of dried squids during storage. This study confirms the biopreservative potential of the SPS-BV in dried seafood products in Thailand.

Keywords: Biopreservative, *Bacillus*, Seafood, Food safety, Dried squid

1. Introduction

Seafood and seafood products has been a relevant part of the community in Thailand because of being important source of proteins, essential amino acids, omega-3 fatty acids, vitamins, and minerals, and providing health benefits in prevention of life-threatening events, e.g. cardiovascular diseases and rheumatic diseases (Venugopal, 2018). Traditional dried seafood-based products are one of the most consumed categories of ready-to-eat (RTE) foods in Thailand due to a busy lifestyle of working individuals, the convenience offered, time-saving, and the little effort required for the preparation. A large part of preparation generally uses wild-caught fish and shellfish as raw materials resulting in the control of certain hazards very difficult in some traditional RTE seafood. As a consequence, consumption of traditional RTE seafood is not risk-free. Seafood represents a high risk of public health with common implication in foodborne outbreak in Thailand accounting for 27.5 and 33.3% of strong-evidence events in 2012 and 2019 (Donla, Junthepa, & Promsiri, 2019; Poonawagul & Jearanaiwongkul, 2012). Traditional RTE seafood products available in a local market in Chon Buri province, Thailand have been reported to contaminate with spoilage bacteria over the acceptable limit of total viable bacteria in processed products (< 5 x 10^5 CFU/g) and cooked products (< 5 x 10^4 CFU/g) imposed by Department of Fisheries

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(2009). Foodborne pathogens, e.g. *Bacillus cereus*, *Escherichia coli*, and *Salmonella* are also existed in the RTE seafood products (Butkhot et al., 2019a; Nimrat et al., 2019; Thungkao & Muangharm, 2008). An improvement of biosafety quality of the RTE seafood products is required through inhibiting the growth of spoilage and pathogenic bacteria. Chemical preservatives have been extensively used in traditional RTE seafood products to eliminate undesirable bacteria. However, a health concern caused by the overuse of chemical additives currently become an important raised issue because some of them are expected to involve in deteriorative health effects, e.g. intoxications, allergies, cancers, hepatotoxicity, and teratogenicity (Zhao et al., 2019). Therefore, there is a great increasing interest in the use of saprophytic bacteria and/or their metabolites as a novel, safe, and food-compatible biopreservative for inhibiting pathogens and extending shelf-life of seafood products.

In the context of food safety, members of *Bacillus* genus, a Gram-positive, aerobic, and endospore-forming bacterium with rod-shaped morphology, have a long history of safe use simultaneously with a potential probiotic application in human and animal foods. Some of the main representatives within *B. subtilis* complex, e.g. *B. amyloliquefaciens*, *B. atrophaeus*, *B. velezensis*, *B. licheniformis*, *B. methylotrophicus*, *B. mojavensis*, *B. siamensis*, *B. subtilis*, *B. tequilensis*, and *B. vallismortis* have been designated as "generally recognized as safe (GRAS)" for human consumption (Lefevre et al., 2017). They have been claimed to be an excellent source of diverse secondary metabolites harboring biopreservative potential, like bacteriocins, lipopeptides, polyketides, siderophores, bacteriocin-like inhibitory substances, and non-ribosomally synthesized peptides (Harwood, Mouillon, Pohl, & Arnau, 2018). Bacteriocins are ribosomal antimicrobial peptides biosynthesized by bacteria for self-defense against the growth of closely related species, and curtain foodborne pathogens through pore formation on the cell surface and interference in cell wall synthesis (Harwood et al., 2018). Bacteriocins produced by *Bacillus* species have been considered as appropriate alternative to chemical preservatives because they represent diverse structural diversity, selectivity towards targeted pathogens owing to both available broad-spectrum and narrow-spectrum activity, stability in a wide conditions of temperature and pH, low allergenic potential, activity at low concentrations, and proteinaceous nature thereby readily being degraded in the gastrointestinal tract (Harwood et al., 2018; Yi, Luo, & Lü, 2018). Currently, only nisin, a pentacyclic bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is commercially available, and legally used as a food additive in Thailand (Notification of Ministry of Public Health, 2018), despite a number of bacteriocins extensively purified and characterized to date.

Selection of bacteriocin-producing species used in food preservation requires close scrutiny in terms of biosafety and beneficial characteristics. Recently, a novel identified strain of *B. velezensis* BUU004 has been confirmed as a promising probiotic and a safe source of biopreservative to prevent foodborne infections of seafood products (Butkhot, Soodsawaeng, Boonthai, Vuthiphandchai, & Nimrat, 2020; Butkhot, Soodsawaeng, Vuthiphandchai, & Nimrat, 2019b). In laboratory study, *B. velezensis* BUU004 has shown strong antibacterial potential against seafood spoilage and pathogenic bacteria, namely *B. coagulans*, *E. coli*, *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* Typhimurium, susceptibility to antibiotics commonly used in human and veterinary clinical therapy, and robust survivability under gastric and bile conditions. The authors also described its non-pathogenic characteristic indicated by low cytotoxicity, and the absence of hemolytic activity and virulence-associated genes: hemolysin genes (*hlyI/clo*, *hlyII* and *hlyIII* homolog), enterotoxin genes (*cytK*, *hblACD* and *nheABC*), hemolytic enterotoxin HBL (*hblABCD*), non-hemolytic enterotoxin NHE (*nheABC*) and cereulide (*cesABCD*). In order to determine if the new *Bacillus* strain intended for use as a source of natural preservative in human foods, the objectives of this study were to investigate antibacterial activity of semi-purified solution containing bacteriocin from *B. velezensis* BUU004 (SPS-BV) against foodborne pathogenic *B. cereus* under *in vitro* environment and food spoilage bacteria in RTE dried, seasoned and crushed squids during storage, and evaluate its effect on physical and chemical qualities (pH, water activity, moisture content, and NaCl content) of the RTE dried squids. In addition, RTE dried seafood products can become potential vehicles of such hazards as pathogen contamination during their journeys through all stages of the preparation. Consequently, bacteriological quality across multiple steps related to preparation of the RTE dried squids was also evaluated**.**

2. Materials and Methods

2.1 Bacteriological evaluation across a series of preparation of dried seasoned and crushed squids

2.1.1 Dried squid preparation

Preparation of dried, seasoned and crushed squids was conducted in a small household facility located in a fishing village in Chon Buri province to simulate a real situation of the dried squid production in Thailand (Nimrat, Soodsawaeng, Rattanamangkalanon, Boonthai, & Vuthiphandchai, 2021). In brief, fresh splendid squids (*Loligo duvauceli*) were caught about 10-km offshore from Bang Sean Beach and its vicinity in the Gulf of Thailand by local fishermen. The preparation was initiated by cutting longitudinally in the center of the abdomen of medium-sized squids using a sharp knife, and removing the head, tentacles, skin, eyes, soft shell, beak and internal organs, and then three-time rinsing with tap water (Figure 1a). The 5-kg squids were marinated in homemade Thai seasoning sauce consisting of chili sauce (1.5 L), sugar (500 g), chili pepper powder (80 g), chopped garlic (10 g), and vinegar (50 mL) for 1 h (Figure 1b). Smooth squids were placed on a steel wire grate (Figure 1c), and sun-dried from 8 am to 4 pm for two successive days (Figure 1d). The semi-dried squids were individually crushed using a rolling machine after which they were grilled on an electric grill with periodically flipping over for 2 min (Figure 1e). The crushed squids were sun-dried again for 1 day prior to package in a plastic bag and storage at room temperature (Figure 1f). Sampling was taken in rinsed squid, marinated squid, first sun-dried squid, grilled squid, crushed squid, and second sun-dried squid to assess bacterial quality.

2.1.2 Microbiological analysis

Bacteriological evaluation across preparation steps of dried, seasoned and crushed squids was performed following a method recommended by US Food Drug Administration [FDA] (1998) with some modifications. A portion (50 g) of samples was homogenized with Butterfield's phosphate-buffered water (450 mL) using a stomacher for 2 min, and successive 10-fold dilution was made in the same buffered solution. A minute volume (0.1 mL) of each dilution was seeded onto Plate Count Agar (Becton BD, Sparks, MD, USA). After incubation at 35 ± 2ºC for 24 h, bacterial colonies were enumerated and calculated as colony forming unit (CFU) of total viable count (TVC) per g of sample. Single colony of isolated bacteria with distinct morphological differences was selected and purified on Trypticase Soy Agar (TSA; Becton BD, Sparks, MD, USA). Identification of bacterial species was made following a classical protocol (Winn et al., 2006) and API test kits (bioMerieux, Marcy I' Etoile, France). All measurements were carried out in triplicate.

2.2 Bacteriocin produced by *B. velezensis* BUU004 and its inhibitory spectrum against *B. cereus*

B. velezensis BUU004 strain was isolated from black tiger shrimp (*Penaeus monodon*) pond sediment and used as probiotic in shrimp culture (Butkhot et al., 2019a). The strain was routinely grown aerobically on a TSA plate at 30ºC. The fresh overnight strain was seeded in Trypticase Soy Broth (TSB; Becton BD, Sparks, MD, USA) at 30ºC, 200 rpm for 18 h. Cell-free supernatant was harvested by centrifugation at 8,000*g* and 4ºC for 10 min, and then partially purified using ammonium sulfate precipitation method (An et al., 2015). The supernatant was saturated with 80% ammonium sulphate subsequently with a constant stirring at 4°C overnight. The precipitates were harvested by centrifugation at 10,000*g*, 4ºC for 30 min and the pellets were dissolved in 50 mM sodium phosphate buffer (pH 7.0) prior to dialysis against a dialysis membrane (1 kDa cutoff, Spectrum Laboratory, Los Angeles, CA, USA) at 4ºC overnight. The obtained SPS-BV was filtered through a 0.45 µm filter (Sartorius, Bedford, MA, USA). The SPS-BV contained bacteriocin displaying proteinaceous nature with molecular weight of ca. 5.75 kDa as evaluated by Tricine SDS-PAGE analysis (Butkhot et al., 2019b). Bacteriocin activity of the SPS-BV was assessed against indicator *B. cereus* TISTR 687 using an agar well diffusion method. Two-fold serial dilution of the SPS-BV was prepared, and the activity was calculated as arbitrary units (AU) per mL on a basis of the formula: $2^n/V$ x 1,000 (µl), where n is the reciprocal of the highest dilution at which bacteriocin produces a clear zone of growth inhibition of the indicator strain, and V is volume of each dilution added into the wells (Butkhot et al., 2019a). The SPS-BV solution (800 AU/mL) was prepared and stored at -80°C for further use.

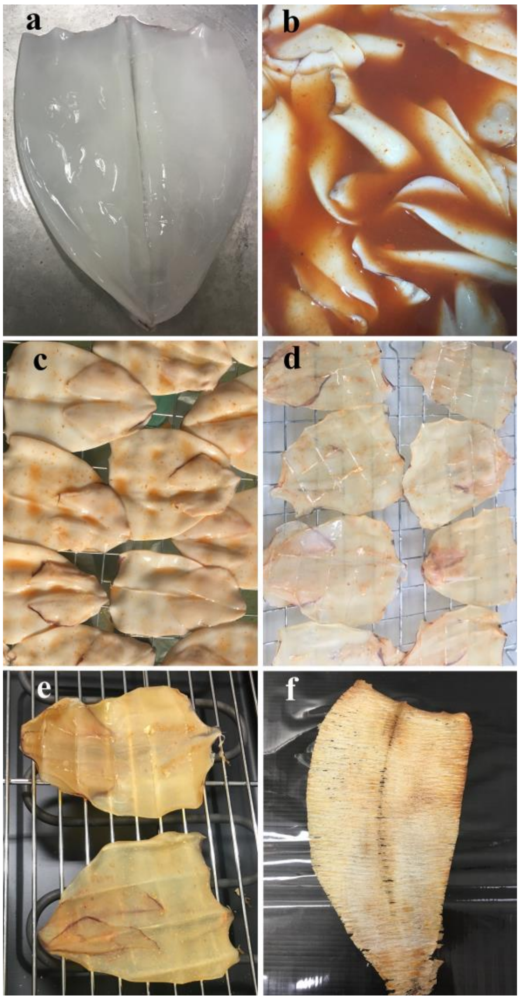

Figure 1. Dried, seasoned and crushed squids during multistep related to preparation processes: (a) rinsed squids, (b) marinated squids in seasoning sauce, (c) squids on a steel wire grate, (d) first sun-dried squids, (e) squids grilled on an electric grill, and (f) dried, seasoned, and crushed squids.

2.2.1 Inhibitory activity against *B. cereus*

Antibacterial activity was investigated using an agar well diffusion assay against *B. cereus* TISTR 687 employed as an indicator (Abdhul et al., 2015). *B. cereus* suspension was prepared at a cell density of 10^8 CFU/mL using the 0.5 McFarland turbidity standard, and then swabbed on the surface of Mueller Hinton agar (Becton BD, Sparks, MD, USA) plates. Thereafter, 6-mm diameter wells were punched by a sterile cork borer. A hundred microliters of the SPS-BV solution (800 AU/mL) was loaded in the wells. All petri dishes were incubated at 35°C for 24 h and diameters of inhibition zone were measured. The experiment was carried out in triplicates.

2.2.2 Scanning electron microscopy

Morphological changes of *B. cereus* treated with the SPS-BV solution were visualized using scanning electron microscopy (SEM). Pathogenic *B. cereus* TISTR 687 was subcultured in TSB 35°C for 18 h to produce the logarithmic-phased cells, and then adjusted to a density of 10^8 CFU/mL using the 0.5 McFarland standard. The adjusted cell suspension was incubated with the SPS-BV solution (800 AU/mL) at 35±1ºC for 20 h. The cell suspension incubated in sterile TSB without any additives was considered as a negative control. The untreated and treated cells of *B. cereus* were centrifuged at 8,000*g*, 4°C for 15 min. Afterwards, the obtained pellets were washed twice with phosphate buffer solution (pH 7.2) and prepared for morphological observations following a conventional protocol (Ullah et al., 2017). Photomicrographs of the cell damages were visualized using a scanning electron microscope (SEM; LEO 1450 VP, ZEISS, Oberkochen, Germany) equipped with an SEM User Interface, LEO-32 software. Bactericidal potential of the SPS-BV solution was determined by spread-plating a portion of the SPS-BV-treated cells on TSA plates, and comparing with untreated cells.

2.3 Antibacterial potential of the SPS-BV in dried, seasoned and crushed squids

2.3.1 Effect of the SPS-BV on food-spoilage bacteria

Nisin consisting of 2.5% active nisin was purchased from Sigma-Aldrich Chemical Co, Darmstadt, Germany. Nisin powder (100 mg) was dissolved in 0.02 N HCl solution to produce nisin solution at a concentration of 10^3 IU/mL. The solution was sterilized by filtering through a 0.45 μm syringe filter, and stored at -20°C for further use (Soodsawaeng, Butkhot, Boonthai, Vuthiphandchai, & Nimrat, 2021). Lactic acid solution containing 80% lactic acid purchased from Sigma-Aldrich, St. Louis, MO, USA was used for preparation of 5 mM lactic acid solution.

The SPS-BV with strong inhibitory activity towards foodborne pathogenic *B. cereus* was employed to control the growth of food-spoilage bacteria in post-prepared dried, seasoned and crushed squids in comparison with commercial preservatives including lactic acid and nisin. First,

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the dried, seasoned and crushed squids were cut into a 2 x 2 cm piece using sterile scissors. The pieces of squid samples were divided into 4 groups including addition of 1) sterile distilled water (control), 2) 5-mM lactic acid, 3) nisin solution (10^3 IU/mL) and 4) the SPS-BV (800 AU/mL). For each treatment, the tested additive (0.1 mL) was slowly introduced onto whole surface of a square piece of the squid sample using an autopipette to allow maximum absorption into the food matrix, and subsequently air-dried for 15 min in a biosafety cabinet (Soodsawaeng et al., 2021). The additive-treated squids were stored separately in a sterile plastic bag according to their treatments. During storage at room temperature (approximately 25°C), the squids were sampled at 15 min and 1, 7, 14, 21 and 28 days of storage for TVC enumeration as aforementioned method.

2.3.2 Effect of the SPS-BV on physical and chemical properties

Changes in physical and chemical qualities, e.g. pH, NaCl content, moisture content and water activity (a_w) of the SPS-BV-treated squids, nisin-treated squids, and lactic-acid-treated squids were evaluated in comparison with the control. Ten grams of the dried squids were homogenized with distilled water (100 mL) in a stomacher bag. pH values were measured using a regular calibrated pH meter (Metrohm; 913 pH meter, Herisau, Switzerland; Karastogianni, Girousi, & Sotiropoulos, 2016). Dried squid samples (10 g) were finely ground using a blender and placed into an aluminium tray. Then, water activity (a_w) was estimated using a water activity meter (Novasina AG; MS1 Set-aw, Lachen, Switzerland; AOAC, 2000) and moisture content was measured using a moisture meter (Sartorius; MA150, Weender Landstraße 94-108 37075, Goettingen, Germany; AOAC, 2000). NaCl content in the dried squids was quantified using a Volhard method (AOAC, 2000). All measurements were conducted in triplicate.

2.4 Statistical analysis

All data are present as mean±standard deviation. Normality of the data was tested and transformation was made when needed. The data were subjected to a two-way analysis of variance (ANOVA) to determine the difference of measured variables. Significant differences were compared using the post-hoc Tukey's test at the level of p<0.05. Statistical analyzes were performed using Minitab version 18.1.0.

3. Results and Discussion

3.1 Bacteriological quality during various steps related to production of dried, seasoned, and crushed squids

Across a series of the preparation, freshly rinsed squids contained low TVC of 1.10±0.00 x 10^3 CFU/g, and then TVC significantly (p<0.05) increased to 3.60±1.41 x 10^3 CFU/g in the squids marinated in seasoning sauce. TVC in the squids significantly (p<0.05) decreased again to 1.80±0.00 x 10^3 CFU/g after first sun-drying. Afterwards, low TVC in the squids ranging from 1.00±0.00 to 4.00±0.00 x 10^2 CFU/g was observed during the phases between grilling and second sun-drying (Figure 2).

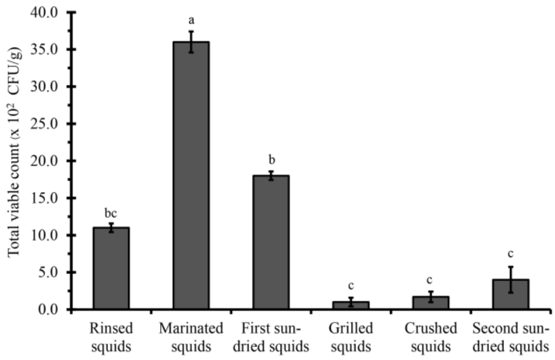

Figure. 2 Bacterial quality across a multistep related to production of dried, seasoned and crushed squids. Letters on bars indicate significant difference (p<0.05).

Bacterial composition of the squids changed across a multistep of preparation. In rinsed squids, two species of Gram-positive cocci were isolated including *Kocuria palustris* (86.4%) and *K. varians* (13.6%; Figure 3a). In marinated squids, bacterial component was changed to spore-forming *Bacillus* species, e.g. *B. circulans* (46.1%), *B. coagulans* (35.8%), and *B. acidocaldarius* (18.1%; Figure 3b). Thereafter, *B. acidocaldarius* remained predominant (53.9%) in first sun-dried squids with the presence of two staphylococci species, namely *Staphylococcus saprophyticus* subsp. *bovis* (27.8%), and *S. pasteuri* (18.3%; Figure 3c). Two *Bacillus* species belonging to *B. brevis* (50%), and *B. marinus* (50%) were predominantly isolated from grilled squids (Figure 3d). Then, crushed squids appeared to be dominated by *B. coagulans* (58.8%) and to a lesser extent by *B. Marinus* (20.6%), and *S.*

saprophyticus subsp. *Bovis* (20.6%; Figure 3e). Lastly, only *B. megaterium* was recovered from second sun-dried squids (Figure 3f).

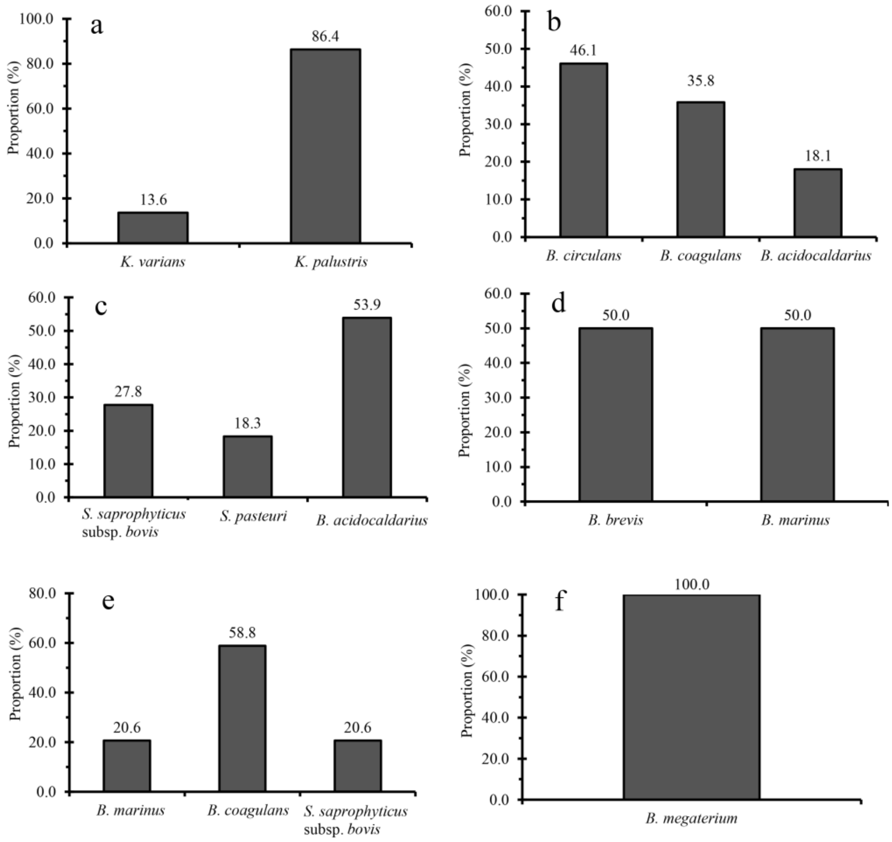

Figure. 3 Composition and abundance of viable bacteria isolated from subsequent phases of the dried squid preparation: (a) rinsed squids, (b) marinated squids, (c) first sun-dried squids, (d) grilled squids, (e) crushed squids, and (f) second sun-dried squids.

A growing demand of traditional RTE seafood products in Thailand has necessitated larger production within the shortest possible time that has placed them at uncertain biosafety status due to a high risk of pathogen contamination. In Thailand, deteriorated bacteriological quality of traditional seafood products has been considered as an important issue in food safety during the past decade (Nimrat et al., 2021). Bacteriological analysis conducted by Butkhot et al. (2019a) has revealed that the biosafety quality of traditional dried seafood-based products in Thailand is relatively poor, indicating a health hazard caused by foodborne diseases. It is widely known that such products can be vehicles of harmful germs causing foodborne illnesses at any stages of preparation. In general, foodborne pathogenic and spoilage bacteria can be introduced into foods at any point of time during the farm-to-fork chain starting from i) primary production (in the farm/sea where animals are raised or caught), ii) preparation processes, iii) transportation, iv) storage, v) distribution, and vi) even the final stage in the consumers' kitchen (Bintsis, 2018). Bacteriological analysis across multiple steps of the food production chain would assist in identifying some potential sources of contamination together with implementation of an effective measure to reduce the health hazard risks of consumers. In the present study, bacterial contamination existed across a series of the preparation of dried, seasoned and crushed squids indicated by the presence of TVC in the ranges of 10^2 – 10^3 CFU/g with abundance of Gram-positive cocci in the genera of *Kocuria* and *Staphylococcus*, and Gram-positive spore-forming *Bacillus* as predominant species. The results were consistent to the recent study conducted by Nimrat et al. (2021). Low TVC of 10^2 CFU/g was observed along a multistep production of dried, seasoned and crushed squid with the presence of *Bacillus*, *Staphylococcus*, and *Micrococcus* as main contaminant bacteria. Despite similar types of processed squids, bacterial composition is comparatively different. In general, bacterial abundance in food products is known to vary largely depending on various factors, e.g. raw material used, physical and chemical properties (like a_w, moisture content, pH), storage environment, and food additives and preservatives used. The present study points out that poor hygienic practices still exist during preparation and production processes of traditional dried seafood products in Chon Buri province. In this study, in the post-rinsed phase of the preparation chain, main bacterial compositions of the rinsed squids were *K. palustris* and *K. varians.* Due to their environmental origin, water may be a source of bacterial contamination in the squids (Khan, Knapp, & Beattie, 2016). Municipal water is commonly used in multiple phases of the preparation chain of dried, seasoned and crushed squids in Thailand. In addition, poor personnel hygiene and inadequate handling may increase the transference possibility of *Kocuria* species from worker's hands to the squids because they are indigenous flora on skins, mucous membranes, and oral cavities of humans (Kandi et al., 2016). Our results also revealed that the most common source of spoilage bacterial contamination was brown seasoning sauce used for squid marination supported by the highest TVC number, and bacterial composition shifted from *Kocuria* to *Bacillus* species. In accordance with Nimrat et al. (2021), predominant bacteria isolated from dried, seasoned and crushed squid marinated in brown seasoning sauce were *Staphylococcus* and *Bacillus* species. This can be speculated that *Bacillus* contamination may be linked to spices and condiments, such as chili pepper powder and garlic used as ingredients in the seasoning sauce. Edible spices and dried herbs are usually susceptible to microbial contamination, especially spoilage bacteria belonging to *Bacillus* genera (Sagoo et al., 2009). Antibacterial potential of spices and other additives in the seasoning sauce may also play a role in inhibiting the growth of *Kocuria* present in the preceding phase of preparation (Curtis, Noll, Störmann, & Slusarenko, 2004). As a consequence, brown seasoning sauce should be supplemented with natural preservatives to reduce bacterial contamination, especially spoilage *Bacillus* species. In the next phase of preparation, the bacterial component was changed again to *B. acidocaldarius* as predominant species with a lesser extent staphylococci species in 1st sun-dried squids. In Thailand, traditional dried seafood products are commonly produced in small factories or household facilities with rudimentary hygienic practices, thereby having a free access of pathogens, flies, insects, rodents, dust, soil, and hazardous chemicals and pollutants (Butkhot et al., 2019a). This may account for such a phenomenon. The presence of *S. saprophyticus* subsp. *bovis* and *S. pasteuri* may also reflect a poor handling manner due to their normal flora on skins, anterior nares and ear canals of humans (Becker, Heilmann, & Peters, 2014). In subsequent stages of preparation, *Bacillus* species become the most frequently isolated bacteria with approximately 80-100% of total bacterial isolates recovered from grilled squids, crushed squids, and second sun-dried squids. Of all bacteria isolated, *Kocuria* spp. has occasionally been a cause of infection usually in patients with immunosuppression including urinary tract infections, cholecystitis, catheter-associated bacteremia, dacryocystitis, canaliculitis, keratitis, peritonitis, brain abscess and meningitis (Kandi et al., 2016). Although heat-stable toxin producing strains of *Bacillus* species, especially *B. cereus*, and *B. subtilis* have been reported as cause of food poisoning linked to consumption of contaminated foods (Logan, 2011), there have been no reports of *Bacillus* species isolated from the squid samples in the present study being associated with foodborne illnesses. However, due to resistance characteristics of the endospores to heat, radiation, preservative, and desiccation, they may survive and multiply to high levels when water becomes available during dried seafood products being stored at room temperature for sale. This can create troublesome problems in commercial dried seafood as changes in textures, production of off-flavors, food defects and organoleptic unacceptability of consumers resulting in a significant financial loss.

In order to secure biosafety from a farm to fork cycle of the traditional dried squid products in Thailand, more stringent measures for hygiene at production facilities are required. The introduction of the microbiological guidance, e.g. Good Hygiene Practices (GHPs), Good Manufacturing Practices (GMPs) and Hazard Analysis Critical Control Point (HACCP) programs in place with the establishment of in-process controls to ensure product integrity,

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rather than reliance on end-product testing for compliance with specifications has been a significant advance in production of safer food products and prevention of outgrowth of pathogenic and spoilage microorganisms. Education programs based on basic principles for microbiological food safety of food handlers can be a straightforward strategy to create biosafety awareness and help to prevent foodborne diseases. Moreover, controlling post-prepared contamination may be one of the key aspects of food safety. In addition to good hygiene practices, biopreservative-based strategy, in particular bacteriocins supplementation has received tremendous attention for controlling the growth of pathogen and spoilage bacteria in post-prepared products to minimize the risk of foodborne illnesses.

3.2 Inhibitory potential against pathogenic *B. cereus* of the SPS-BV

The SPS-BV exhibited strong antibacterial activity towards foodborne pathogenic *B. cereus* with an inhibition zone of 14.0±0.2 mm (Figure 4). It had bactericidal activity observed by no growth of the SPS-BV-treated pathogen on the TSA plate whereas the untreated cells grew well on the medium (data not shown). Its mode of action against *B. cereus* was evaluated using SEM analysis. Normal intact cell walls were observed in untreated cells of *B. cereus* (Figure 4a). Conversely, the SPS-BV severely destroyed the cellular structure of *B. cereus* cells evident from pore formation along with extremely flattened cells (Figure 4b). This may be indicative of intracellular fluid efflux.

B. cereus can easily contaminate dried seafood products (Butkhot et al., 2019a) and causes two types of gastrointestinal diseases: diarrhea and emesis (Huang, Flint, & Palmer, 2020). It is difficult to eliminate *B. cereus* contamination in food products due to its heat tolerance of the endospores and easy growth of the vegetative cells at a broad range of temperatures. In the present study, the SPS-BV exhibited antibacterial potential against food poisoning strain of *B. cereus*. The results were corroborated with a study reported by Perumal, Yao, Kim, Kim, and Kim (2019). Culture supernatant containing a bacteriocin BacBS2 produced by *B. velezensis* BS2 isolated from Korean fermented seafood showed strong inhibitory spectrum towards various species of pathogenic Gram-positive and negative bacteria, especially *B. cereus* and *Listeria monocytogenes* in laboratory media. Similarly, Lee and Chang (2017) claimed that *B. subtilis* SN7 strain produced a novel bacteriocin, namely mejucin, with high antibacterial activity against various pathogens, especially *B. cereus*. Then, the SN7 strain was used as a starter culture in chongkukjang, a Korean

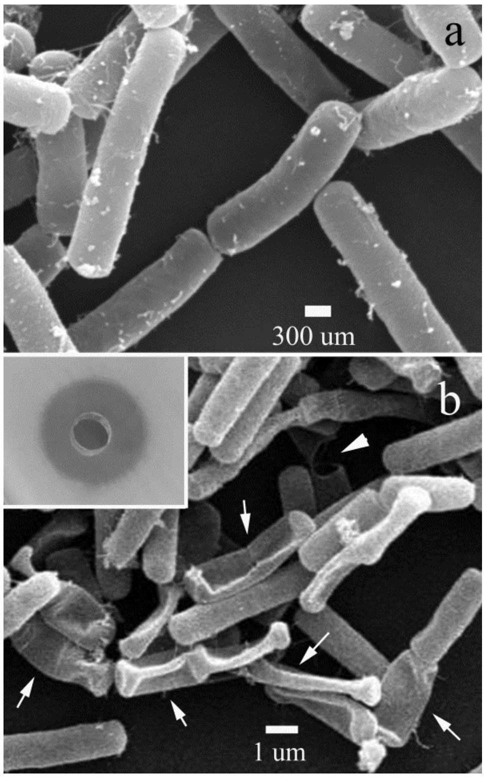

Figure. 4 SEM microphotographs of (a) untreated *Bacillus cereus* representing regular and intact cell walls, and (b) the-SPS-BV-treated cells representing pore formation (➤) and ghost cells with cytoplasmic material completely lost (↑) with inset showing inhibitory activity against *B. cereus* of the SPS-BV based on an agar well diffusion technique.

fermented soybean product, and exhibited a complete growth inhibition of experimentally inoculated *B. cereus* cells during fermentation and 6-month refrigerated storage. SEM analysis revealed that the SPS-BV had bactericidal activity against *B. cereus* through cell lysis supported by pore formation along with severe deconstruction of the cell structure. It seems that mode of action of the SPS-BV in this study may be related to a fluidifying effect on the cellular structure that facilitates depolarization, disintegration of the protective outer membrane, increased membrane permeability, change in the proton motive force, formation of transient pores, efflux of intracellular fluids, and eventually cell death (Malanovic & Lohner, 2016).

The results confirmed antibacterial potential of the SPS-BV for controlling the growth of pathogenic and spoilage bacteria in food products.

3.3 Effect of the SPS-BV on food-spoilage bacteria, and physical and chemical properties of dried, seasoned, and crushed squids

3.3.1 Effect on food-spoilage bacteria in dried, seasoned and crushed squids

During 28-day storage at room temperature, TVC in the untreated squids were in the ranges of 1.75±0.50 - 6.00±1.63 x 10^2 CFU/g (Figure 5). Addition of the SPS-BV was as effective as nisin for controlling the growth of food-spoilage bacteria. TVC in the SPS-BV-treated squids and nisin-treated squids remained comparatively stable ranging from undetectable level to 2.00±0.82 x 10^2 CFU/g, which were significantly (p<0.05) lower than those of the control. Lactic acid could control the growth of spoilage bacteria by 21 days of storage, and thereafter lactic acid-treated dried squids exhibited a significant ($p < 0.05$) increase in

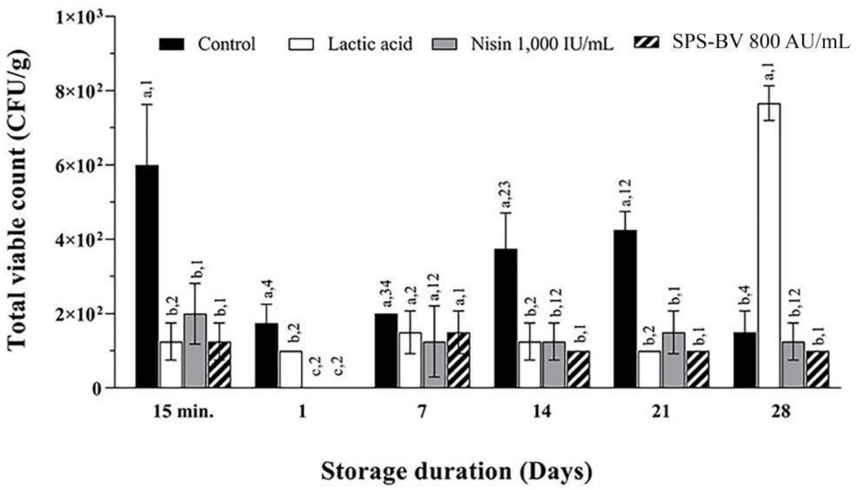

Figure. 5 Effect of the tested additives on food-spoilage bacteria in dried, seasoned, and crushed squids during storage at room temperature for 28 days. Means with superscript letters at each sampling period indicate significant difference ($p < 0.05$) among treatments. Means with superscript numbers in each treatment indicate significant difference ($p < 0.05$) over time.

TVC, compared to those of the SPS-BV-treated squids and nisin-treated squids at day 28 of storage (Figure 5). Our results indicated that supplementation of the SPS-BV was as effective as commercial nisin for controlling food spoilage bacteria in dried, seasoned and crushed squids during storage at room temperature. Similar results were reported by Fangio and Fritz (2014). Crude extract of a bacteriocin-like substance produced by a harmless strain of *B. cereus* P9 was administrated in food samples. As a result, it showed a strong antibacterial activity observed by significant reductions in mesophilic and psychrotrophic aerobic bacteria counts in fresh beef and *B. cereus* counts in lettuce (*Lactuca sativa* L.) under refrigerated storage. Tumbarski et al. (2020) also studied the potential use of a novel bacteriocin from *B. methylotrophicus* strain BM47 for biopreservation and improvement of the storage life of fresh tomato (*Solanum lycopersicum* L.) juice. The bacteriocin produced by *B. methylotrophicus* strain BM47 retained low TVC population in fresh tomato juice, especially in combination with pasteurization, compared to the control group during 24-day chilled storage. Reduced TVC population in the RTE dried squids in the present study is possibly expected due to the SPS-BV activity through cell lysis as mentioned previously. Lactic acid has been classified as GRAS by the U.S. Food and Drug Administration. It widely used as a promising alternative of food additive for inhibition of pathogenic and spoilage bacteria in human products due to its antimicrobial activity, flavor enhancer, antioxidant potential, prevention of lipid oxidation by decreasing the pro-oxidative effect of NaCl (Paelinck & Szczepaniak, 2005). In the present study, lactic acid demonstrated an immediate antibacterial effect, and prolonged period of bacteriostasis with low TVC population for up to 21-day storage, but thereafter, its inhibitory activity seemed significantly reduced as supported by a pronounced increase in TVC population at day 28 of storage. The results were in contrast to the previous report by Greer and Dilts (1995). Pork with fat tissue was immersed in a 3% lactic acid solution at 55°C. Lactic acid immediately eliminated spoilage bacteria on the pork to below detectable level and no growth of artificially-inoculated meat-spoilage bacteria, e.g. *Pseudomonas fragi* and *Brochothrix thermosphacta* were observed throughout 15-day storage at 4°C. Conversely, the spoilage bacterial numbers were approximately 8 log cycle in water-treated pork. Discrepancies on the ability of lactic acid for controlling the growth of spoilage bacteria on food products could be related to food types, treatment conditions, and sensitivity of indigenous flora to the organic acids. Inactivation of lactic acid activity is possibly associated, in part with the fact that lactic acid concentration progressively decreases during storage to a level at which inhibitory potential against spoilage bacterial cells is faded out in food matrix due to its high reactivity of the two adjacent functional groups: acid and alcohol, in the molecules (Komesu et al., 2017).

3.3.2 Effect on physical and chemical properties of dried, seasoned and crushed squids

Lactic acid, nisin, and the SPS-BV influence physical and chemical changes of dried, seasoned and crushed squids in a different way. Addition of either nisin or the SPS-BV resulted in a significant ($p < 0.05$) increase in pH values (5.79±0.03 and 5.81±0.04) at 15-min post-exposure, and afterwards, similar pH values were recorded in nisin-treated, SPS-BV-treated squids, and the control during 28-day storage, except at 21-day storage of nisin-treated squid (Figure 6a). Moisture contents of nisin-treated, and SPS-BV-treated squids ranged from 13.2±1.4 - 20.8±1.0%, and 13.2±0.5 - 22.0±0.3%, respectively, which were significantly lower than that of the control (13.5±1.0 - 26.5±0.0%) throughout 28-day storage, except at day 14 and 21 of storage (Figure 6b). Similar to moisture content, a_w values of the dried squids significantly ($p < 0.05$) decreased in the ranges of 0.571±0.001 - 0.739±0.004 and 0.568±0.003 - 0.707±0.000 following addition of nisin and the SPS-BV, respectively in comparison with the control (0.570±0.004 - 0.738±0.003) during 28-day storage, except at 14-day post-storage (Figure 6c). All tested additives had no effect on NaCl content in the dried squid samples during storage (Figure 6d).

Physical and chemical quality is an important parameter to indicate the food quality and its change can affect sensorial quality and consumer acceptability. In this study, significant reductions of moisture content and a_w were observed simultaneously with no effects on pH and NaCl content following addition of the SPS-BV in the RTE dried squids. Generally, a_w is related to water present in food in free form necessary for microbial growths, chemical-biochemical reactions, rates of deteriorative reactions, and physiochemical properties of food products (Fontana, 2000). a_w of the SPS-BV-treated squids significantly decreased in comparison with the control during storage. Similarly, Arief, Jenie, Suryati, Ayuningtyas, and Fuziawan (2012) reported that addition of 0.3% bacteriocin solution from *Lactobacillus plantarum* 2C12 caused a reduced a_w in beef ball while a_w of the untreated beef ball obviously increased during 6-day refrigerated storage. In fact, a decrease in a_w is a desirable environment to inhibit the growth of spoilage bacteria in dried food products. In this context, the significant reduction in a_w of the SPS-BV-treated squids may account for such decrease in TVC population in the present study. Relationship between the SPS-BV addition and a change in a_w is currently unknown in the dried squid samples. A significant reduction in a_w of the SPS-BV-treated squids may be explained by an osmotic dehydration process as observed in several products following

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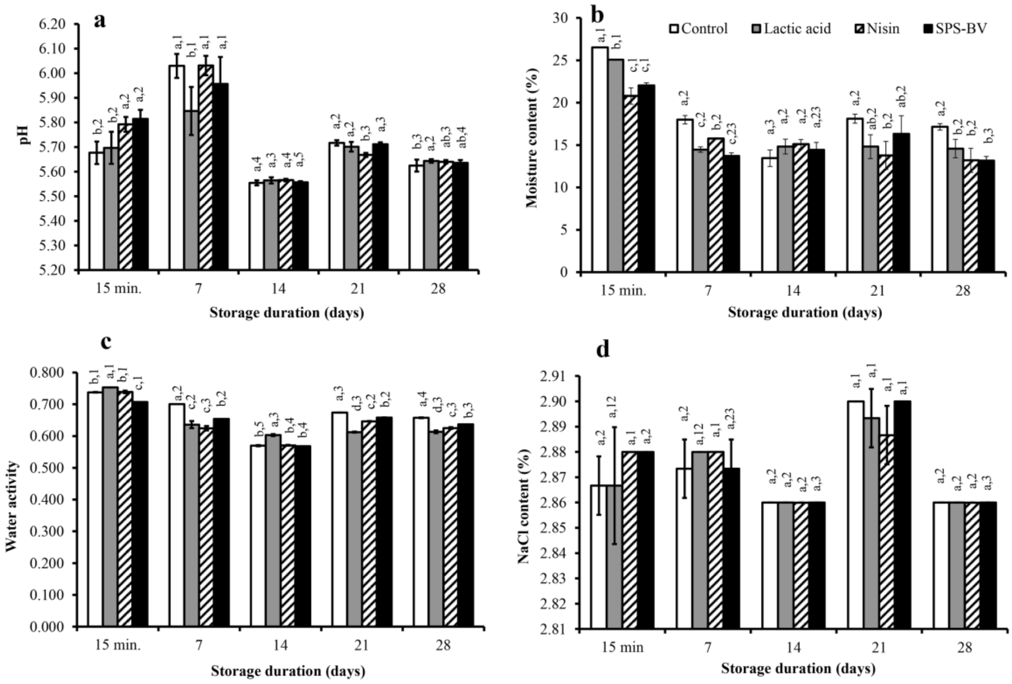

Figure. 6 Physical and chemical changes: (a) pH, (b) moisture content, (c) water activity, and (d) NaCl content of dried, seasoned and crushed squids following addition of the tested additives during storage for 28 days. Means with superscript letters indicate significant difference ($p < 0.05$) among treatments. Means with superscript numbers indicate significant difference ($p < 0.05$) over time.

addition of solutes into foods such as NaCl, sucrose, glucose, and inverted sugar. The mechanisms involve a water-soluble solute flow into the food matrix and an outflow of free water from the product into the environment resulting in increased diffusion of active antibacterial agents for improving biosafety quality (Erkmen & Bozoglu, 2016). Despite strong antibacterial activity, a_w of the SPS-BV-treated squids was over allowable limit at 0.6 of dried RTE squids distributed in Thailand (Thai Industrial Standards Institute, 2010). This indicates a need for an effective alternative strategy for administration of the SPS-BV in RTE dried squids while maintaining its antibacterial activity. Edible films derived from natural products are such examples of technological application, which can be a promising method serving as carriers and providing a controlled release of antimicrobials over an extended period of time without deteriorative effect on physical and chemical quality of foods. Moisture content influences food characteristics, e.g. physical appearance, texture, taste, shelf-life, and even resistance to spoilage caused by bacterial activity. Even though moisture content of the SPS-BV treated squids obviously reduced during storage in comparison with the control, there is no regulatory standard for RTE squid products imposed by the food administration agency of Thailand. A change in pH of foods may affect its color and sensorial quality. Such increase in pH reflects meat spoilage due to protein decomposition and the formation of alkaline compounds. However, pH of the SPS-BV-treated squids did not significantly differ with the control during storage, suggesting a protective activity against proteolytic reactions of the additive.

Although nisin is accepted as an effective and safe antibacterial peptide with FDA-approved and GRAS status and commonly used as food preservative in Thailand and other countries, such limitation of nisin usage in commercial foods is compromised by its expensiveness, lack of inhibitory actions against Gram-negative bacteria, and development of nisin-resistant pathogenic strains (Zhou, Fang, Tian, & Lu, 2014). Our results confirm an equal antibacterial activity of nisin and the SPS-BV for controlling the growth of food spoilage bacteria in RTE dried squids. This suggests that application of the SPS-BV can meet demand for natural preservatives and provide financial benefits by reducing the costs of treatment in RTE dried seafood products. However, an additional study focused on organoleptic quality is required to ensure the consumer acceptability of dried, seasoned and crushed squid in terms of sensorial properties.

4. Conclusions

Across a series of the preparation, TVC populations between rinse and 2nd sun-dry phases were in the ranges of 10^2 - 10^3 CFU/g. The most common source of spoilage bacteria contamination was brown seasoning sauce used for squid marination supported by the highest TVC population and diversity. Main contaminant bacteria isolated from prepared dried squids were *Bacillus*, *Kocuria* and *Staphylococcus.* The SPS-BV was as effective as commercial nisin in terms of controlling food spoilage bacteria in dried, seasoned and crushed squids during storage without effects on pH and NaCl content. However, significant reductions in moisture content and a_w were produced following addition of the SPS-BV in the dried squids. Mode of mechanisms of the SPS-BV against spoilage and pathogenic bacteria was through cell lysis supported by pore formation along with severe deconstruction of the cell structure. The results suggest application potential of the SPS-BV as biopreservative in RTE dried seafood products.

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Conflict of Interest

No conflict of interest declared.

ORCID

Pornpimon Soodsawaeng:
https://orcid.org/0000-0001-7793-7684
Traimat Boonthai:
https://orcid.org/0000-0002-8251-6486
Verapong Vuthiphandchai:
https://orcid.org/0000-0003-3311-4939
Subuntith Nimrat:
https://orcid.org/0000-0001-9327-2408

Ethical Approval

All procedures in this study were in accordance with the ethical standards approved by the Research Unit Committee, Faculty of Science, Burapha University (September 1, 2018).

Burapha University Animal Care and Use Committee

Publication Ethic

The submitted manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere.

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