Research Article

Journal of Applied Science and Emerging Technology (JASET)
Published by Faculty of Applied Science, KMUTNB, Thailand
doi: 10.14416/JASET.KMUTNB.2023.02.002
Vol. 22, No. 2 [2023]: e246628

Incorporation of Bacteriocin Produced by *Bacillus velezensis* BUU004, Plant Extracts and Their Combination for Controlling Food Spoilage Bacteria in Dried Squid Snack Product

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

[1]Biological Science Program, [2]Department of Aquatic Science and

[3]Department of Microbiology Faculty of Science, Burapha University, Thailand

ABSTRACT

Traditional seafood-based product is one of popular cuisines in Thailand and easily susceptible to contamination of pathogenic and spoilage bacteria, thereby representing a serious risk of foodborne infection. A novel effective alternative technology is required for enhancing biosafety quality along with shelf-life extension of seafood products. This study aimed to evaluate preservative potential of naturally occurring compounds on spoilage bacteria in dried squid snack. The squids were divided into 4 treatments including supplementation of 1) sterile distilled water (control), 2) a semi-purified solution containing bacteriocin from *B. velezensis* BUU004 (SPS-BV; 800 AU/mL), 3) a mixture of lemongrass and hot pepper extracts (MLH; 160 mg/mL) and 4) a combination of the SPS-BV (800 AU/mL) and MLH (160 mg/mL), and then stored in a refrigerated condition for 28 days. Strategies of the tested-additive administration included a single addition at beginning of trial and addition of the additives every 14-day of storage. A single addition of the three tested additives was shown to be unlikely to decrease total viable count (TVC) in dried squid snack. In contrast, the antibacterial activity was more evident in dried squids supplemented with the SPS-BV or the novel combination with every 14-day addition observed by 21.8% and 14.1% TVC decrement, respectively. Our results indicate that the SPS-BV and the novel combination have a preservative potential for controlling the growth of spoilage bacteria in dried squid snack. However, due to their degenerative antibacterial activity in dried squids during storage, a development of intelligent active and controlled release packaging technologies that can deliver the antimicrobials into the seafood systems over prolonged periods is required to improve safety of seafood products in Thailand.

KEYWORDS: *Bacillus velezensis*, Food safety, Food-spoilage bacteria, Seafood, Chon Buri

*Corresponding Author: subunti@buu.ac.th

Received: 12/11/2021; **Revised:** 13/03/2023; **Accepted:** 10/04/2023

1. INTRODUCTION

Traditional seafood-based product is one of popular Thai cuisines widely distributed in tourist attraction areas and retail stores in coastal provinces in eastern and southern Thailand. Annual consumption has clearly increased because of its unique tastes, flavors and textures, and abundance of essential nutrients. It has occupied nearly 7% of the total seafood production of Thailand equivalent to approximately 1,781,100 tons in 2017 (Fisheries Statistics of Thailand, 2019). It is mostly produced in small commercial processing factories and household traditional plants located along the Gulf of Thailand and shores of the Andaman Sea, especially in Chon Buri province with 88 factories and traditional plants (Fisheries Statistics of Thailand, 2019). Most of the traditional seafood products (ca. 80%) are produced for domestic consumption. It is generally known that traditional seafood-based product is vulnerable to spoilage due to contamination of pathogenic and spoilage microorganisms, e.g. *Staphylococcus* spp., *Bacillus* spp., *Pantoea* spp., *Micrococcus* spp., *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* (Thungkao & Muangharm, 2008; Butkhot et al., 2019a; Nimrat et al., 2019). In general, contamination of food-borne pathogenic and spoilage bacteria in food products can easily occur at any point of a farm to table cycle, including primary production (in the farm/sea where animals are raised or caught), food processing, storage, and distribution (Bintsis, 2018). In Chon Buri provinve, a public health issue of traditional processed squid products is alarming due to 86.5% samples compiled between 2002 and 2019, containing viable bacterial count over the acceptable limits imposed by Thailand and international food administration agencies (Nimrat et al., 2021). Such a phenomenon represents a serious risk of food-borne infection, like diarrhea, gastritis, and food poisoning illnesses, and results in shortening of the shelf-life and economic loss as well as prompts development of an effective alternative technology for controlling undesirable bacteria.

Synthetic preservatives and chemical additives have legally permitted as an antimicrobial agent in food products for extending the shelf life and securing the microbiological quality. However, some chemicals are suspected to cause intoxications, allergies, cancers, hepatotoxicity, teratogenicity, and other degenerative syndromes, when long-term ingestion of the excessive dose (Zhao et al., 2019). In recent years, consumers demand for convenient, safe, and lightly preserved foods with free of chemical additives that permit shelf-life extension and secure biosafety of seafood-based products. As a consequence, biopreservative-based technology of naturally occurring substances, e.g. plant extracts and bacterial-derived compounds have become a topic of interest to control the growth food spoilage and pathogens in food systems. Bacteriocin, a ribosomally synthesized peptide with antibacterial activity against closely-related and non-related bacteria through bactericidal mechanisms, has much received attention as natural preservatives in food products. Recently, we observed that a novel bacteriocin produced by *Bacillus velezensis* BUU004 (800 AU/mL) had a great potential use as biopreservative due to its distinct inhibitory activity against foodborne Gram-positive and Gram-negative bacteria in *in vitro* environment and dried seafood model (Butkhot et al., 2019a, b). Similarly, extracts from lemongrass (*Cymbopogon citratus* (DC) Stapf.) and hot pepper (*Capsicum frutescens* L.) have been also used for food preservation with antibacterial activity (Soodsawaeng et al., 2021). In addition, several reports have revealed that combination of the antibacterial agents exhibits stronger efficient potency towards food spoilage and pathogenic microorganisms together with prolonging the shelf life of seafood products (Grande et al., 2007; Field et al., 2015; Zhao et al., 2019). Therefore, the objective of this study was to ascertain biopreservative potential of bacteriocin produced by *B. velezensis* BUU004, lemongrass and hot pepper extracts (MLH), and their combination for controlling food spoilage bacteria in dried, seasoned and crushed squids, simply called dried squid snack during storage.

2. MATERIALS AND METHODS

2.1 Preparation of herb extracts

Lemongrass (stems) and hot pepper (fruits) were supplied from a local botanical garden in Chon Buri Province. The extraction was conducted according to the procedure explained by Soodsawaeng et al. (2021). The herb material was dried and ground into a powder using a blender (Fig. 1a, 2a). Ethanol (95%) was added to each powder sample at a ratio of 1:10 of material to extractant. The solvent mixture was filtered after a constant agitation at 30ºC, 120 rpm, for 72 h (Fig. 1b, 2b). Then, the filtrate was concentrated at 40ºC using a rotary evaporator (Buchi R-215, Flawil, Switzerland; Fig. 1c, 2c). A stock solution (160 mg/mL) was prepared by dissolving the crude ethanolic extract in 35% ethanol and stored in an amber bottle until use.

2.2 Bacteriocin production from B. velezensis BUU004

A probiotic strain of *B. velezensis* BUU004 has been confirmed as a non-pathogen with a potential safe use as a source of biopreservative in seafood products (Butkhot et al., 2019b, 2020). The stock culture of *B. velezensis* BUU004 was maintained at -80 ºC in Trypticase Soy Broth (TSB; Becton BD, Sparks,

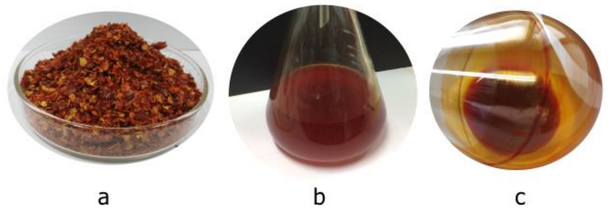

Figure 1 Hot pepper (*Capsicum frutescens* L.): a) ground hot pepper, b) ethanolic extract, and c) evaporated hot pepper extract

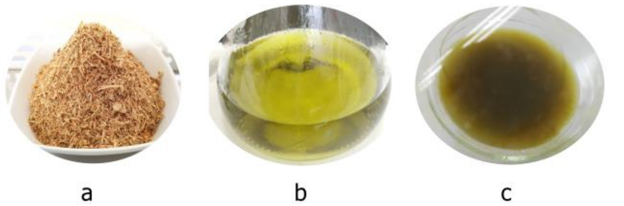

Figure 2 Lemongrass (*Cymbopogon citratus* (DC) Stapf.): a) ground lemongrass, b) ethanolic extract, and c) evaporated lemongrass extract

Maryland, USA) with 15% glycerol addition. The strain was subcultured twice on Trypticase Soy Agar (TSA; Becton BD, Sparks, Maryland, USA) to produce working culture. *B. velezensis* BUU004 was grown in TSB in a shaking incubator at 30 ºC, 200 rpm for 18 h. The cells were removed by centrifugation at 8,000 g and 4ºC for 10 min. Bacteriocin from the culture supernatant was precipitated using simple purification procedure (Butkhot et al., 2019b). Briefly, ammonium sulphate was added into the supernatant at 80% saturation and allowed to settle overnight at 4°C. The precipitated protein was harvested by centrifugation at 10,000 g, 4ºC for 30 min, and then dissolved in 50 mM sodium phosphate buffer (pH 7.0). Thereafter, the bacteriocin-containing suspension was dialysed against a dialysis membrane (1 kDa cutoff, Spectrum Laboratory, Los Angeles, CA, USA) at 4ºC overnight. Evaluation of bacteriocin activity of the semi-purified solution containing bacteriocin from *B. velezensis* BUU004 (SPS-BV) was performed using a well diffusion method against *B. cereus* TISTR 687 used as indicator (Butkhot et al., 2019b). Bacteriocin activity was calculated as arbitrary units (AU) per mL and the SPS-BV solution was stored at -80ºC until use.

2.3 Effect of the SPS-BV, the MLH and their combination on food-spoilage bacteria in dried squid snack

Biopreservative potential of the tested additives was evaluated following Butkhot et al. (2019a) method. Dried squid snack products were purchased from a retail store at Nong Mon Market, Chon Buri province after which they were produced in a small household facility located in a fishing village following a preparation method described by Soodsawaeng et al. (2023). The squid samples were prepared by cutting into a small piece of 2 × 2 cm. The samples were divided into 4 treatments as follows: addition of 1) sterile distilled water (control), 2) the SPS-BV (800 AU/mL), 3) the MLH (160 mg/mL) and 4) a combination of the SPS-BV (800 AU/mL) and the MLH (160 mg/mL), and then air-dried for 15 min in a biosafety cabinet at room temperature (approximately 25ºC). For each treatment, the tested additives (0.1 mL) were slowly supplemented onto the entire surface of a piece of the squid samples. The squid samples with tested additives were kept in a sterile plastic bag under refrigerated condition (2-4ºC) for 28 days. At day 14 and 28 of storage, half of the samples from each treatment were re-introduced with their respective additives. Bacterial evaluation was conducted at 15 min and 1, 7, 14, 21 and 28 day post-inoculation.

2.4 Enumeration of total viable count (TVC) and bacterial identification

TVC in the squid samples was enumerated based on a spread plate method (US FDA, 1998) with some modifications. The squid sample (5 g) was mixed with 0.1% (w/v) peptone water (45 mL) and homogenized using a stomacher. Then, the homogenate was 10-fold diluted and an aliquot (0.1 mL) from each dilution was spread-plated onto Plate Count Agar (Becton BD, Sparks, Maryland, USA). All petri dishes were incubated at 35±2ºC for 24 h. All bacterial colonies were enumerated and calculated as colony forming unit (CFU) per g of sample. All measurements were carried out in triplicate.

Isolated bacteria with distinct colony morphology were identified based on biological characteristics and 16S rRNA gene sequencing analysis. The genomic DNA used as template for PCR amplification was extracted from a freshly grown bacterial culture using an InstaGene Matrix (Bio-rad Laboratories, Inc., USA) following manufacturer's instruction. The primer pair used in this study was 27F 5′ (AGA GTT TGA TCM TGG CTC AG) 3′ and 1492R 5′ (TAC GGY TAC CTT GTTACG ACT T) 3′ (Kaur et al., 2017). The PCR reaction mixture contained genomic DNA (20 ng) in a 30 µl reaction mixture using an EF-TaqDNA Polymerase (SolGent, Yuseong-gu, Daejeon, Korea). The PCR reaction was performed using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., PTC-0240, Foster City, California, USA). The following PCR program was employed including initial activation at 95 °C for 2 min, and 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min with a final cycle of 72 °C for 10 min. The PCR products were purified using a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was conducted using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. All nucleotides sequences were blasted against the sequences within the EzTaxon-e server (https://www.ezbiocloud.net/; Kim et al., 2012). The sequences are deposited in GenBank (Accession no. MZ298612 – MZ298619).

2.5 Data analysis

Data are expressed as mean±standard deviation. Bacterial counts were transferred to log values after normal distribution and homoscedasticity were tested. Data were analyzed using a two-way analysis of variance (ANOVA), and followed by the post-hoc Tukey's test to identify any difference between the control and treatments. Significance was defined at a level of $p<0.05$. The statistical analyses were conducted using Minitab version 18.1.0.

3. RESULTS

3.1 TVC in dried squid snack with/without the tested additives

TVC of the pre-treated squid samples was 9.1±0.6×103 CFU/g. At 15-min post-exposure, TVC in squids with addition of the MLH, the SPS-BV, and a combination of MLH and SPS-BV were 5.7±0.8×103, 4.4±0.2×103, and 7.2±0.7×103 CFU/g, respectively, which were significantly ($p<0.05$) lower than that of the control (9.1±1.5×103 CFU/g). However, TVC in the SPS-BV-treated and the combination-treated squids comparatively increased at 28-day storage supported by 16.3% and 11.2% increment, respectively. When the supplements were added every 14-day, the most effective strategy against TVC in the squid samples was SPS-BV administration with 21.8% decrement, and followed by supplementation of a combination of MLH and SPS-BV due to 14.1% decrement at the end of refrigerated storage (Table 1).

3.2 TVC in dried squid snack with/without the tested additives

Heterotrophic bacteria isolated from untreated squids throughout 28-day refrigerated storage were composed of 7 Gram-positive strains: *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Bacillus velezensis* strain 1, *B. tequilensis*, *B. licheniformis*, *B. velezensis* strain 2, *B. subtilis* subsp. *inaquosorum* and *B. safensis* subsp. *safensis* while *B. subtilis* subsp. *stercoris* was recovered in untreated squids until day 1 of storage. *S. saprophyticus* subsp. *saprophyticus* was eliminated at day 14, 28 and 21 of storage in the squids treated with the MLH, SPS-BV, and their combination, respectively. *B. subtilis* subsp. *stercoris* was absent in the MLH-treated, the SPS-BV-treated, and the combination-treated squids at 15-min, 1-day, and 1-day post exposure, respectively. When the three additives were added every 14 days, similar results were observed due to the absence of *S. saprophyticus* subsp. *saprophyticus* and *B. subtilis* subsp. *stercoris* during storage (Table 2).

4. DISCUSSION

In the recent years, special attentions on food preservation have been paid to naturally derived substances as a novel biopreservative in food products. Bacteriocins produced by lactic acid bacteria have been long used in fresh and processed foods without degenerative health problems and deleterious effects on

Table 1 TVC (CFU/g) in dried squid snack with/without the MLH, the SPS-BV, and their combination during 28-day refrigerated storage

Storage duration (days)	Distilled water	MLH	SPS-BV	MLH + SPS-BV	MLH (every 2-w addition)
15-min	$9.1 \pm 1.5 \times 10^{3\ a,B}$	$5.7 \pm 0.8 \times 10^{3\ bc,C}$	$4.4 \pm 0.2 \times 10^{3\ c,C}$	$7.2 \pm 0.7 \times 10^{3\ b,BC}$	$4.2 \pm 0.3 \times 10^{3\ c,C}$
1	$9.6 \pm 0.3 \times 10^{3\ a,B}$	$6.8 \pm 0.7 \times 10^{3\ b,BC}$	$8.8 \pm 0.7 \times 10^{3\ a,B}$	$7.8 \pm 1.6 \times 10^{3\ ab,AB}$	$8.3 \pm 0.6 \times 10^{3\ ab,A}$
7	$9.6 \pm 0.6 \times 10^{3\ a,B}$	$9.1 \pm 1.2 \times 10^{3\ a,A}$	$1.1 \pm 0.1 \times 10^{4\ a,A}$	$9.5 \pm 0.9 \times 10^{3\ a,A}$	$4.3 \pm 0.2 \times 10^{3\ b,C}$
14	$1.1 \pm 0.1 \times 10^{4\ a,A}$	$8.7 \pm 0.5 \times 10^{3\ c,AB}$	$1.0 \pm 0.1 \times 10^{4\ ab,A}$	$6.3 \pm 0.8 \times 10^{3\ d,BC}$	$8.9 \pm 0.6 \times 10^{3\ bc,A}$
21	$8.1 \pm 0.3 \times 10^{3\ a,B}$	$9.3 \pm 0.9 \times 10^{3\ a,A}$	$8.1 \pm 0.9 \times 10^{3\ a,B}$	$5.7 \pm 0.3 \times 10^{3\ b,C}$	$6.4 \pm 0.7 \times 10^{3\ b,B}$
28	$9.1 \pm 0.3 \times 10^{3\ a,B}$	$5.6 \pm 1.3 \times 10^{3\ b,C}$	$5.2 \pm 0.2 \times 10^{3\ bc,C}$	$7.9 \pm 0.7 \times 10^{3\ a,AB}$	$4.4 \pm 0.4 \times 10^{3\ bc,C}$
Change (%)	-0.3	-2.3	16.3	11.2	2.8

Means with superscript lowercase letters indicate significant difference ($P < 0.05$) among treatments. Means with superscript uppercase letters indicate significant difference ($P < 0.05$) over time.

organoleptic properties of foods. Likewise, bacteriocins from *Bacillus* species have also currently increasing importance in food industry because of their wide spectrum of antibacterial activity, distinct structural diversity, low cytotoxicity, and stability under hostile environments, thereby remaining their activity during thermal processing cycle of foods (Abriouel et al., 2011; Butkhot et al., 2019b). In the present study, a single addition of the SPS-BV was unlikely to decrease TVC in dried squids during 28-day refrigerated storage. It is widely known that bacteriocins are easily degraded by indigenous and/or microbial proteolytic enzymes, and inactivated by their binding to the food components, like proteins, carbohydrates and fats in food products (Aasen et al., 2003; Stergiou et al., 2006). These reasons may be explained why the SPS-BV was inactive towards TVC in the dried squids in the present study. Antibacterial activity against TVC of the SPS-BV supplemented every 14-day was more evident than a single addition in the squids, especially inhibition of *S. saprophyticus* subsp. *saprophyticus* and *B. subtilis* subsp. *stercoris*. The lower antagonistic potency in dried squids when a single administration was applied is possibly associated with a reduced concentration of bacteriocins during storage to a level at which no inhibitory activity can be produced (Ghalfi et al., 2007). As a consequence, we suggested administration of the SPS-BV in dried seafood products every 14-day during storage. Our results were accordant with several previous reports. Liu et al. (2017) demonstrated that bacteriocins, namely amylolysin and amylocyclicin produced by *B. velezensis* LS69 was shown to strongly inhibit Gram-positive and Gram-negative pathogenic bacteria, e.g. *B. cereus*, *B. thuringiensis*, *S. aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, *E. coli* DH5α, and *Pseudomonas putida*. Similarly, in our recent study, the SPS-BV was effectively active against food-spoilage and foodborne pathogens including *S. aureus*, *B. cereus*, *B. coagulans*, *L. monocytogenes*, *Micrococcus luteus*, *E. coli*, *E. coli* O157:H7 and *Salmonella* Typhimurium in *in vitro* experiment (Butkhot et al., 2019b). The inhibition of bacterial growth observed in this study is probably a consequence of cell lysis, pore formation, and severe cell membrane deconstructions induced by the SPS-BV (Butkhot et al., 2019a; Soodsawaeng et al., 2023). In general, bacterial cell mortality caused by bacteriocins produced by *Bacillus* genera is produced through destabilization of the cellular structure, destroy membrane integrity, efflux of cytoplasmic fluids, and disruption of cellular activities, e.g., energy production and membrane transport (Abriouel et al., 2011).

The MLH produced undesirable results indicated by unchanged TVC number in dried squids during storage. Similarly, the MLH was shown to have low inhibitory activity against food spoilage bacteria in food system trial (Soodsawaeng et al., 2022). It is widely known that a cocktail of mixed terpenes and terpenoids, e.g., citral, neral, isoneral, geranial, isogeranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and elemol, are present predominantly in lemongrass extract/essential oil (Mukarram et al., 2022) while the

Table 2 Bacterial composition of dried squid snack with/without the tested additives during 28-day refrigerated storage

Treatments	Storage duration (days)	*S. saprophyti*	*B. velezensis*	*B. tequilensis*	*B. lichenifor*	*B. subtilis* ssp.	*B. velezensis*	*B. subtilis* ssp.	*B. safensis*
Distilled water	15 min	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+
	7	+	+	+	+	−	+	+	+
	14	+	+	+	+	−	+	+	+
	21	+	+	+	+	−	+	+	+
	28	+	+	+	+	−	+	+	+
MLH	15 min	+	+	+	+	−	+	+	+
	1	+	+	+	+	−	+	+	+
	7	+	+	+	+	−	+	+	+
	14	−	+	+	+	−	+	+	+
	21	−	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+
SPS-BV	15 min	+	+	+	+	+	+	+	+
	1	+	+	+	+	−	+	+	+
	7	+	+	+	+	−	+	+	+
	14	+	+	+	+	−	+	+	+
	21	+	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+
MLH + SPS-BV	15 min	+	+	+	+	+	+	+	+
	1	+	+	+	+	−	+	+	+
	7	+	+	+	+	−	+	+	+
	14	+	+	+	+	−	+	+	+
	21	−	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+
MLH (every 14-day addition)	15 min	+	+	+	+	−	+	+	+
	1	+	+	+	+	−	+	+	+
	7	+	+	+	+	−	+	+	+
	14	−	+	+	+	−	+	+	+
	21	−	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+
SPS-BV (every 14-day addition)	15 min	+	+	+	+	+	+	+	+
	1	+	+	+	+	−	+	+	+
	7	−	+	+	+	−	+	+	+
	14	−	+	+	+	−	+	+	+
	21	−	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+
MLH + SPS-BV (every 14-day addition)	15 min	+	+	+	+	+	+	+	+
	1	+	+	+	+	−	+	+	+
	7	−	+	+	+	−	+	+	+
	14	−	+	+	+	−	+	+	+
	21	−	+	+	+	−	+	+	+
	28	−	+	+	+	−	+	+	+

+: found, −: not detectable

major compositions of ethanolic chili extract include phenolic compounds and capsaicinoids, particularly capsaicin, dihydrocapsaicin, protocatechuic acid, p-coumaric acid, cinnamic acid, and ferulic acid (Menezes et al., 2022). In general, mode of action of active components in herb extracts against bacterial cells includes sequential inhibition of biochemical reactions, the intervention of various protective enzymes, destabilization of the bacterial membrane structure by causing a change in the permeability of the cytoplasmic membrane, and leakage of a variety of molecules and ions (Cava-Roda et al. 2021). However, low antibacterial efficacy of the herb extracts in the present study is likely to involve in the volatile properties of herb extracts and the reduced concentrations of active ingredients in the herb extracts during 28-day storage (Abdollahzadeh et al., 2014). Inhibitory spectrum of plant-based substances can be potentiated with the presence of bacterioicins in food systems. However, no synergistic activity against bacterial growth was produced in the present study observed by a combination of the SPS-BV and the MLH having antibacterial potential behind the SPS-BV in the chilled-stored dried squids. The results were in contrast to previous report by Shahbazi et al. (2016). A combined addition of a commercial nisin with essential oil (0.1-0.2%) from *Ziziphora clinopodioides* caused an obvious reduction in *E. coli* O157:H7 numbers in raw beef patties during 9-day refrigerated storage. A bioengineered derivative nisin V in combination with low concentration of either carvacrol or trans-cinnamaldehyde also acted synergistically to reduce viable cells of *L. monocytogenes* in laboratory media, chocolate milk drink and chicken noodle soup (Field et al., 2015). The volatile properties of herb extracts and the reduced concentrations of active compounds in the herb extracts during storage may account for compromised biopreservative potential of the novel combination, resulting in no synergy observed in the present study. However, a decreased antibacterial efficacy of the combined additives may not be originated from only one factor. Intrinsic factors of the seafood, interaction among active compounds, and environmental parameters may influence bacterial sensitivity to the additives. Reduced number of spoilage bacteria caused by addition of the SPS-BV would help to extend shelf-life and maintain food quality at a desired level so that maximal benefits and nutrition values can be achieved. However, additional study related to biochemical assessment and texture profile analysis should be established in order to confirm its compatibility in food products.

5. CONCLUSION

Our results suggest that the SPS-BV had stronger antibacterial potential for controlling the growth of food spoilage bacteria than the MLH, and the novel combination in dried squid snack during storage, in particular, when the additive was added every 14-day. Addition of the SPS-BV could eliminate some spoilage bacteria, e.g. *S. saprophyticus* subsp. *saprophyticus* and *B. subtilis* subsp. *stercoris* in dried squid snack during storage. However, its antibacterial activity was inactivated during chilled storage observed by low antagonistic potential in dried squids when a single administration was applied. As such, it is essential to develop an effective method with desirable physicochemical properties that can serve as carriers and provide a controlled release of antimicrobials over an extended period of time, such as edible films derived from natural products in order to create a food-compatible and natural preservative for technological applications in food industry and improve biosafety quality of traditional seafood-based products in Thailand.

ACKNOWLEDGEMENTS

This study was sponsored by the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0109/2556) to S. Nimrat and P. Soodsawaeng and the Research Grant of Burapha University through National Research Council of Thailand (Grant no. 44/2561). We thank Biological Science Program, and Department of Microbiology, Faculty of Science, Burapha University, Thailand for providing experimental equipment and facilities.

REFERENCES

Aasen, I. M., Markussen, S., Moretro, T., Katla, T., Axelsson, L., & Naterstad, K. (2003). Interactions of the bacteriocins sakacin P and nisin with food constituents. *International Journal of Food Microbiology*, *87*(1-2), 35-43. https://doi.org 10.1016/s0168-1605(03) 00047-3

Abdollahzadeh, E., Rezaei, M., & Hosseini, H. (2014). Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. *Food Control*, *35*(1), 177-183. https://doi.org/10.1016/j.foodcont.2013.07.004

Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2011). Diversity and applications of Bacillus bacteriocins. *FEMS microbiology reviews*, *35*(1), 201-232. https://doi.org/10.1111/j.1574-6976.2010.00244.x

Bintsis, T. (2018). Microbial pollution and food safety. *AIMS Microbiology*, *4*(3), 377-396.

Butkhot, N., Soodsawaeng, P., Samutsan, S., Chotmongcol, K., Vuthiphandchai, V., & Nimrat, S. (2019). New perspectives for surveying and improving Thai dried seafood qualities using antimicrobials produced by *Bacillus velezensis* BUU004 against foodborne pathogens. *ScienceAsia*, *45*(2), 116-126. https://doi.org/10.2306/scienceasia1513-1874.2019.45.116

Butkhot, N., Soodsawaeng, P., Vuthiphandchai, V., & Nimrat, S. (2019). Characterisation and biosafety evaluation of a novel bacteriocin produced by *Bacillus velezensis* BUU004. *International Food Research Journal*, *26*(5), 1617-1625.

Butkhot, N., Soodsawaeng, P., Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2020). Properties and safety evaluation of *Bacillus velezensis* BUU004 as probiotic and biopreservative in seafood products. *Southeast Asian Journal of Tropical Medicine and Public Health*, *51*(2), 201-211.

Cava-Roda, R., Taboada-Rodríguez, A., López-Gómez, A., Martínez-Hernández, G. B., & Marín-Iniesta, F. (2021). Synergistic antimicrobial activities of combinations of vanillin and essential oils of cinnamon bark, cinnamon leaves and cloves. *Foods*, *10*, 1406. https://doi.org/10.3390/foods10061406

Field, D., Daly, K., O'Connor, P. M., Cotter, P. D., Hill, C., & Ross, R. P. (2015). Efficacies of nisin A and nisin V semipurified preparations alone and in combination with plant essential oils for controlling *Listeria monocytogenes*. *Appllied and Environmental Microbiolology*, *81*(8), 2762-2769. https://doi.org/10.1128/AEM.00070-15

Fisheries Statistics of Thailand. (2019). Fisheries statistics of Thailand 2017. Fisheries Statistics Analysis and Research Group, Fisheries Development Policy and Strategy Division, Department of Fisheries, Ministry of Agriculture and Cooperatives, No. 9/2019. https://www4.fisheries.go.th/local/file_document/ 20200714161650_1_file.pdf

Ghalfi, H., Benkerroum, N., Doguiet, D. D. K., Bensaid, M., & Thonart, P. (2007). Effectiveness of cell-adsorbed bacteriocin produced by *Lactobacillus curvatus* CWBI-B28 and selected essential oils to control *Listeria monocytogenes* in pork meat during cold storage. *Letters in Applied Microbiology*, *44*(3), 268-273. https://doi.org/10.1111/j.1472-765X.2006.02077.x

Grande, M. J., Lopez, R. L., Abriouel, H., Valdivia, E., Ben Omar, N., Maqueda, M., Canamero, M. M., & Galavez, A. (2007). Treatment of vegetable sauces with enterocin AS-48 alone or in combination with phenolic compounds to inhibit proliferation of *Staphylococcus aureus*. *Journal of Food Protection*, *70*(2), 405-411. https://doi.org/10.4315/0362-028x-70.2.405

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H., Yi, H., Won, H., & Chun, J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal of Systematic and Evolutionary Microbiology*, *62*(Pt_3), 716-721. https://doi.org/10.1099/ijs.0.038075-0

Liu, G., Kong, Y., Fan, Y., Geng, C., Peng, D., & Sun, M. (2017). Whole-genome sequencing of *Bacillus velezensis* LS69, a strain with a broad inhibitory spectrum against pathogenic bacteria. *Journal of Biotechnology*, *249*, 20-24. https://doi.org/10.1016/j.jbiotec.2017.03.018

Menezes, R. D. P., Bessa, M. A. D. S., Siqueira, C. D. P., Teixeira, S. C., Ferro, E. A. V., Martins, M. M., Cunha, L. C. S., & Martins, C. H. G. (2022). Antimicrobial, antivirulence, and antiparasitic potential of *Capsicum chinense* Jacq. extracts and their isolated compound capsaicin. *Antibiotics*, *11*(9), 1154. https://doi.org/10.3390/antibiotics11091154

Mukarram, M., Choudhary, S., Khan, M. A., Poltronieri, P., Khan, M. M. A., Ali, J., Kurjak, D., & Shahid, M. (2022). Lemongrass essential oil components with antimicrobial and anticancer activities. *Antioxidants*, *11*(1), 20. https://doi.org/ 10.3390/antiox11010020

Nimrat, S., Butkhot, N., Samutsan, S., Chotmongcol, K., Boonthai, T., & Vuthiphandchai, V. (2019). A survey in bacteriological quality of traditional dried seafood products distributed in Chon Buri, Thailand. *Science & Technology Asia*, *24*(4), 102-114.

Nimrat, S., Soodsawaeng, P., Rattanamangkalanon, N., Boonthai, T., & Vuthiphandchai, V. (2021). Biosafety, bacteriological quality and strategy of biopreservative administration for controlling spoilage bacteria in Thai traditional dried seafood products. *African Journal of Microbiology Research*, *15*(10), 512-521.

Shahbazi, Y., Shavisi, N., & Mohebi, E. (2016). Effects of Ziziphora clinopodioides essential oil and nisin, both separately and in combination, to extend shelf life and control *Escherichia coli* O157:H7 and *Staphylococcus* aureus in raw beef patty during refrigerated storage. *Journal of Food Safety*, *36*(2), 227-236. https://doi.org/10.1111/jfs. 12235

Soodsawaeng, P., Butkhot, N., Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2021). Synergistic antibacterial effects of bacteriocin produced by *Bacillus velezensis* BUU004 and medicinal plant extracts against *Escherichia coli* and *Salmonella* Typhimurium in dried, crushed and seasoned squid. *International Food Research Journal*, *28*(4), 654-663.

Soodsawaeng, P., Rattanamangkalanon, N., Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2022). Preservative potential of Thai herb extracts combined with bacteriocin from *Bacillus velezensis* BUU004 for controlling food spoilage and pathogenic bacteria in dried crushed seasoned squids. *Science and Technology Asia*, *27*(1), 74-88.

Soodsawaeng, P., Rattanamangkalanon, N., Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2023). Bacteriocin from *Bacillus velezensis* BUU004 as a seafood preservative: antibacterial potential, and physical and chemical qualities of dried, seasoned, and crushed squids. *Suan Sunandha Science and Technology Journal*, *10*(1), 105-119.

Stergiou, V. A., Thomas, L. V., & Adams, M. R. (2006). Interactions of nisin with glutathione in a model protein system and meat. *Journal of Food Protection*, *69*(4), 951-956. https://doi.org/10.4315/0362-028X-69.4.951

Thungkao, S., & Muangharm, S. (2008). Prevalence of *Bacillus* spp. and *Bacillus cereus* in dried seasoned squid products. *In Proceedings of 46th Kasetsart University Annual Conference: Agro-industry*, (pp. 138-146). Kasetsart University, Bangkok.

US Food Drug Administration. (1998). *Bacteriological Analytical Manual* (8^{th} ed.). Maryland: Association of Official Analytical Chemists International.

Zhao, S., Li, N., Li, Z., He, H., Zhao, Y., Zhu, M., Wang, Z., Kang, Z. & Ma, H. (2019). Shelf life of fresh chilled pork as affected by antimicrobial intervention with nisin, tea polyphenols, chitosan, and their combination. *International Journal of Food Properties*, *22*(1), 1047-1063. https://doi.org/10.1080/10942912.2019.1625918