# Antibacterial Activity of Plant Extract: the Role of Lemongrass and Chili Extracts, a Bacteriocin from *Bacillus velezensis* BUU004, and Their Combination to Control *Staphylococcus aureus* in Dried, Seasoned and Crushed Squid

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**Abstract**

*Staphylococcus aureus* is the leading cause of foodborne bacterial diseases worldwide, and exists in a wide variety of food products in Thailand, particularly dried seafood products. This study evaluated the inhibitory potential against *S. aureus* of mixed extracts of lemongrass and hot chili (MLC), bacteriocin, which is produced by *Bacillus velezensis* BUU004, and their combination, and their effects on physicochemical quality of dried, seasoned, and crushed squids. Dried squid samples were inoculated with *S. aureus* suspension and then divided into 4 treatments: addition of 1) distilled water (control), 2) a partially-purified solution containing bacteriocin from *B. velezensis* BUU004 (PPS-BV; 800 AU/mL), 3) an MLC (160 mg/mL), and 4) a combination of the PPS-BV and MLC. Squid samples were maintained at 2 storage temperatures: either at 4°C or room temperature (approximately 25°C) for 28 days. Two administration regimes of the tested additives were applied: a single addition at the onset of the experiment, and a 14-day addition at 14 and 28 days of storage. Strong anti-staphylococcal activity was observed in the dried squids treated with MLC, and a combination of the PPS-BV and MLC during 28-day storage at both conditions, in particular when the 14-day addition was applied. In contrast, the PPS-BV was ineffective against *S. aureus* in the dried squid samples. The MLC and the combination had bactericidal potency by destroying the bacterial cell walls and cell lysis. Supplementation of the MLC caused a significant reduction ($p<0.05$) in pH simultaneously with a significant increase ($p<0.05$) in the water activity of the dried squid during storage. This study suggests a potential use of MLC as a biopreservative for controlling the growth of *S. aureus* in dried seafood products.

**Keywords:** Food safety, Seafood, *Bacillus*, Bacteriocin, Biopreservation

## Introduction

Seafood and seafood products are enriched sources of proteins, omega-3 fatty acids, vitamins, and other essential nutrients. In Thailand, traditional dried seafood products are a popular seafood choice with an estimated production of 1,781,100 tons in 2017 (Fisheries Statistics of Thailand, 2019). The consumption of seafood and seafood-based products is of interest and concern because of their easy contamination by indigenous flora present in marine environments and foodborne pathogens, e.g. *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* at any point of a farm to table cycle (Butkhot et al., 2019a; Nimrat et al., 2019). *S. aureus* is a well-known versatile foodborne pathogen that causes a wide range of infections from mild disturbances to life-threatening diseases and can survive in a variety of environments (Hennekinne, De Buyser, & Dragacci, 2012). The bacterium is frequently isolated from several types of foods, namely meat, dairy products, fish, retail foods, ready-to-eat foods, and even seafood products (Castro, Silva, & Teixeira, 2018). Enterotoxigenic *S. aureus* was detected in fishery products and fish processing

factory workers in India ( Simon & Sanjeev, 2007), and marine shrimp products in Iran ( Arfatahery, Mirshafiey, Abedimohtasab, & Zeinolabedinizamani, 2015). In Thailand, the prevalence of *S. aureus* was approximately 15% in traditional dried seafood products distributed in Chon Buri province (Nimrat et al., 2019). In Bangkok, the Food Sanitation Division also reports *S. aureus* as a common contaminant in several foods (ca. 16%) sold in flea markets, restaurants, supermarkets, and street food stalls in 2018 behind *E. coli* (57%; Food Sanitation Division, 2020). The presence of *S. aureus* in food products reflects poor hygiene standards in food preparation and processing environments, and inadequate food handling hygiene in cooked and processed foods because it is an indigenous flora found on the skin and mucous membranes of humans (Castro et al, 2018).

It is widely known that *S. aureus* causes an acute gastrointestinal disease terminologically called staphylococcal food poisoning following the ingestion of staphylococcal enterotoxins ( Hennekinne et al., 2012; Castro et al, 2018). Common symptoms of foodborne intoxication are acute gastroenteritis, e.g. nausea, vomiting, abdominal cramps, diarrhea, and fever that appear rapidly within 2-6 hours after the consumption of the staphylococcal-enterotoxin contaminated foods ( Hennekinne et al., 2012). Based on epidemiological surveillance in 2019, staphylococcal enterotoxins were involved in 25.75% of food poisoning outbreaks in Thailand corresponding to the second rank of pathogenicity after acute gastroenteritis caused by *Clostridium perfringens* ( 46.45%; Bureau of Epidemiology, 2019). However, the food poisoning events associated with the staphylococcal enterotoxins were likely to have been underreported due in part to misdiagnosis, improper sample collection and laboratory examination, lack of seeking medical advice by the affected persons, and lack of routine surveillance of clinical stool specimens for *S. aureus* and its enterotoxins (Kadariya, Smith, & Thapaliya, 2014). Given these problems, an effective measure for controlling the growth of *S. aureus* in food products is necessary to minimize the occurrence of staphylococcal food-poisoning outbreaks.

Currently, there is a growing trend to form a natural preservation technology to respond to the demands of health-conscious consumers for convenient, safe, and nutritious food products. To secure food safety, natural substances, e.g. bacteriocins and plant-based extracts, are considered suitable alternatives to chemical preservatives for inhibition of pathogens and extension of product shelf-life. In our recent trial, a bacteriocin from *Bacillus velezensis* BUU004 showed the potential for antibacterial therapy and food preservation due to its low cytotoxicity, the absence of enterotoxin production, and easy degradation in the gastrointestinal tract (Butkhot, Soodsawaeng, Vuthiphandchai, & Nimrat, 2019b). A combination of mixed herb extracts (MLC) from lemongrass (*Cymbopogon citratus* (DC) Stapf.) and hot chili (*Capsicum frutescens* L.), together with the bacteriocin from *B. velezensis* BUU004 (PPS-BV), demonstrated antibacterial potential against spoilage bacteria with no degenerative effects on the sensorial qualities of dried, seasoned, and crushed squid (Soodsawaeng, Rattanamangkalanon, Boonthai, Vuthiphandchai, & Nimrat, 2022).

The objectives of this study, therefore, were to determine the biopreservative potential of PPS-BV, the MLC, and their combination for controlling *S. aureus* growth, and investigate the effects of the tested additives on physicochemical quality of dried, seasoned, and crushed squid during storage.

## Methods and Materials

### Preparation of herb extracts

Herb materials of two plant species, lemongrass (stems) and hot chili (fruits), were collected from a local botanical garden in Chon Buri Province. Herb extraction was carried out using a conventional technique (Soodsawaeng et al., 2022). The herbs were washed, rinsed with distilled water, and dried in shade. The dried herbs were ground using a blender, and the fine powders were submerged in a round bottom flask containing ethanol (95%) at a ratio of 1:10 of material to extractant with agitation at 120 rpm, 30°C, for 72 hr. The liquid extracts were separated from the solid residues by filtration using Whatman no. 1 filter paper before evaporation at 40°C under reduced pressure using a rotary evaporator ( Buchi R-215, Flawil, Switzerland). Crude extract solution (160 mg/mL) was prepared by dissolving the ethanolic herb extract in 35% ethanol and kept at −20°C for further study.

### Bacteriocin production from *B. velezensis* BUU004

*B. velezensis* BUU004 is a non-pathogenic bacteriocin-producing strain with the potential to be used as a safe source of biopreservatives in seafood products (Butkhot et al., 2019b; Butkhot, Soodsawaeng, Boonthai, Vuthiphandchai, & Nimrat, 2020). The bacteriocin-producing strain was cultured in Trypticase Soy Broth ( TSB; Becton BD, Sparks, MD, USA) in a shaking incubator at 30°C, 200 rpm for 18 hr. The cell suspension was centrifuged at 8,000 *g* and 4ºC for 10 min and then, the bacterial cell pellets were discarded. Ammonium sulphate was added slowly to the cell-free supernatant until reaching 80% saturation. The product was harvested by centrifugation at 10,000 *g*, 4ºC for 30 min after which the solution was allowed to settle overnight at 4°C. A partially-purified solution containing bacteriocin from *B. velezensis* BUU004 (PPS-BV) was prepared by dissolving the product in 50 mM sodium phosphate buffer (pH 7.0) and then dialysed against a dialysis membrane (1 kDa cutoff, Spectrum Laboratory, Los Angeles, CA, USA) at 4°C overnight. The antibacterial activity of the PPS-BV was assessed against indicator *B. cereus* TISTR 687 based on a well-diffusion technique and expressed as arbitrary units (AU) per mL as detailed by Butkhot et al. (2019b). The PPS-BV solution (800 AU/mL) was prepared and stored at -80°C until used.

### Effect of the PPS-BV, the MLC, and their combination on foodborne pathogenic bacteria in dried, seasoned and crushed squids

A foodborne pathogenic strain of *S. aureus* ATCC 25923 was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained at the Department of Microbiology, Faculty of Science, Burapha University. The strain was pre-cultured on Trypticase Soy Agar (TSA; Becton BD, Sparks, MD, USA) overnight in a 35°C incubator. The suspension was prepared by suspending a loopful culture of the pathogen in 0.85% normal saline solution, and the bacterial concentration was estimated by optimal density (OD) analysis at 600 nm using a spectrophotometer. Simultaneously, a standard curve was calculated and used to determine the number of cells in the suspension at a level of $10^4$ CFU/mL based on the OD value.

The antibacterial potential of the tested additives against *S. aureus* was investigated using dried, seasoned, and crushed squid as a food model (Butkhot et al., 2019a). A 2 x 2 cm piece of the *S. aureus*-free squid product was made using sterile scissors and then thoroughly inoculated with the prepared *S. aureus* suspension (0.5 mL) and air-dried in a biosafety cabinet for 15 min to allow maximum adhesion to the food matrix. The squid samples were then treated with 0.1 mL of one of these additives: 1) the PPS-BV (800 AU/mL), 2)

the MLC (160 mg/mL) and 3) a combination of the PPS-BV (800 AU/mL) and MLC (160 mg/mL). A squid sample prepared with distilled water was considered as a control. After air-drying for 15 min, the squid samples for each batch were separately stored in a sterile plastic zip-lock bag at either 4°C or room temperature (approximately 25°C). During the trial, the dried squid samples were taken for pathogen enumeration at 15 min post-additive introduction and 1, 7, 14, 21 and 28 days of storage. Additionally, at 14 and 28 days of storage, half of the samples from each batch were re-treated with their respective additives.

**Enumeration of *S. aureus***

*S. aureus* was counted based on the most probable number (MPN) method (US Food Drug Administration, 1998). Briefly, the dried squid (2 g) were 10-fold diluted in Butterfield's phosphate-buffered dilution water, at each defined sampling interval, and thoroughly homogenized using a stomacher. A 1-mL aliquot from each dilution was added into a series of three MPN tubes containing TSB supplemented with 10% NaCl and 1% sodium pyruvate. All tubes were incubated at 35°C ± 1°C for 48 ± 2 hr. Tubes with turbidity were streaked onto Baird Parker Agar (Becton BD) with egg yolk and tellurite supplements and incubated at 35°C ± 1°C for 48 ± 2 hr. Suspicious colonies of *S. aureus* (grey to black colonies with/without clear zone and opaque zone surrounding colonies) were subjected to a coagulase test and other selected biochemical and morphological tests for confirmation. The MPN table was employed to calculate the approximate number of bacteria per gram.

**Scanning electron microscopy**

Scanning electron microscopy was used to visualize any morphological changes of the *S. aureus* cells treated with the tested additives. The results showed that the PPS-BV demonstrated low antibacterial potential against *S. aureus*; so the MLC (160 mg/mL) and a combination of the PPS-BV (800 AU/mL) and MLC (160 mg/mL) were selected for SEM observation. The indicator *S. aureus* was grown in TSB at 35°C for 18 hr and the logarithmic-phased cells were adjusted to a density of $10^8$ CFU/mL using the 0.5 McFarland turbidity standard. The pathogen suspension was incubated with either the MLC or the combination of the PPS-BV and MLC at 35±1°C for 20 hr. The treated *S. aureus* cells were centrifuged at 8,000 rpm, 4°C for 10 min, washed twice with Phosphate Buffer Saline (PBS; pH 7.2) and prepared for SEM observation (Butkhot et al., 2019a). The cell suspension with sterile TSB added was considered as a control. The ultrastructure of the *S. aureus* cells was evaluated using a high-resolution scanning electron microscope (LEO 1450 VP, ZEISS, Oberkochen, Germany). Simultaneously, the untreated and treated *S. aureus* suspensions were spread-plated onto Trypticase Soy Agar plates (Becton BD) to evaluate the bactericidal activity of the tested additives.

**Physicochemical properties of dried, seasoned, and crushed squids**

Due to similar inhibitory potential against *S. aureus* being produced in the dried squid samples treated with the MLC or a combination of the PPS-BV and MLC, only the dried squid with *S. aureus* inoculation and the MLC was selected for physiochemical evaluation to reduce complications and the cost of the additive preparation. The physiochemical properties of the squid samples were determined in three sample groups: the squids without any additive (control), the water-added squids, and the squids with *S. aureus* inoculation and the MLC. pH values were evaluated using a pH meter (Metrohm; 913 pH meter, Herisau, Switzerland; Karastogianni, Girousi, & Sotiropoulos, 2016). Water activity ($a_w$) was measured using a water activity meter (Novasina AG; MS1 Set-aw, Lachen, Switzerland; AOAC, 2000). All measurements were performed in triplicate.

**Data analysis**

The values of the physical properties of the squid samples were expressed as mean±standard deviation. Statistical analyzes were performed using Minitab version 18.1.0. The data were subjected to a two-way analysis of variance (ANOVA) to determine the difference between measured variables. Significant differences were compared using the *post hoc* Tukey's test at $p<0.05$.

## Results

**Effect of the PPS-BV, the MLC, and their combination on *S. aureus* viability**

A high survival rate of *S. aureus* (> 1,100 MPN/g) was found in the control squid samples throughout the 28 days of refrigerated storage. The PPS-BV was ineffective in controlling the growth of *S. aureus* as evidenced by the high viability (≥ 1,100 MPN/g) observed during the 28-day storage period (Table 1). On the contrary, the MLC and the combination of the PPS-BV and MLC appeared effective against *S. aureus* in those squid samples which were indicated by a progressive reduction in the viability until dropping to 43 and 15 MPN/g, respectively at the end of storage period (Table 1). When a 14-day addition of the tested additives was conducted, similar to the single addition, high inhibitory potential against *S. aureus* was noticed both in the presence of the MLC and the combination of the PPS-BV and MLC in the dried squid samples where the pathogen numbers were below the detectable limit (< 3.0 MPN/g) at 21 and 28 days of storage (Table 1).

**Table 1** *S. aureus* number (MPN/g) in dried, seasoned, and crushed squids with the addition of tested additives during 28-day refrigerated storage

| Treatments | Storage duration (days) | | | | | |
|---|---|---|---|---|---|---|
| | 15 min | 1 | 7 | 14 | 21 | 28 |
| Distilled water | >1,100 | >1,100 | >1,100 | >1,100 | >1,100 | >1,100 |
| MLC | 1,100 | >1,100 | 460 | 460 | 460 | 43 |
| PPS-BV | >1,100 | >1,100 | >1,100 | 1,100 | 1,100 | 1,100 |
| MLC + PPS-BV | >1,100 | >1,100 | >1,100 | 210 | 75 | 15 |
| MLC (every 14-day addition) | >1,100 | >1,100 | 460 | 93 | <3.0 | <3.0 |
| PPS-BV (every 14-day addition) | >1,100 | >1,100 | >1,100 | 1,100 | 460 | 43 |
| MLC + PPS-BV (every 14-day addition) | >1,100 | 1,100 | >1,100 | 93 | 3.6 | <3.0 |

During storage at room temperature in a plastic bag, the survival pattern of *S. aureus* in the dried squid samples treated with all the tested additives was different, compared to that in the refrigerated samples. The viability of *S. aureus* in the control squid samples progressively decreased from >1,100 MPN/g at 15-min post-exposure to an undetectable level (< 3 MPN/g) at 14 days of storage (Table 2). A similar pattern of *S. aureus* survival was produced in the dried squid samples with a single addition and 14-day addition of the PPS-BV which was observed by a progressive reduction of *S. aureus* numbers from >1,100 MPN/g at 15-min storage to 23 MPN/g at 7-day post-storage. Similar inhibitory activity against *S. aureus* was found in the dried squid samples treated with a combination of the PPS-BV and MLC through either a single addition or

every 14-day addition. *S. aureus* in the dried squid samples with the combined additive under both administration regimes was ≥ 1,100 MPN/g at the onset of the trial to 93 and < 3 MPN/g at 1 and 7 days of storage (Table 2).

**Table 2** *S. aureus* number (MPN/g) in dried, seasoned and crushed squids with the addition of tested additives during storage at room temperature in a plastic bag

| Treatments | Storage duration (days) | | | | | |
|---|---|---|---|---|---|---|
| | 15 min | 1 | 7 | 14 | 21 | 28 |
| Distilled water | >1,100 | 1,100 | 150 | <3.0 | <3.0 | <3.0 |
| MLC | >1,100 | 29 | <3.0 | <3.0 | <3.0 | <3.0 |
| PPS-BV | >1,100 | 240 | 23 | <3.0 | <3.0 | <3.0 |
| MLC + PPS-BV | 1,100 | 93 | <3.0 | <3.0 | <3.0 | <3.0 |
| MLC (every 14-day addition) | 1,100 | 21 | <3.0 | <3.0 | <3.0 | <3.0 |
| PPS-BV (every 14-day addition) | >1,100 | 240 | 23 | <3.0 | <3.0 | <3.0 |
| MLC + PPS-BV (every 14-day addition) | >1,100 | 93 | <3.0 | <3.0 | <3.0 | <3.0 |

**Effect on the structural architecture of *S. aureus* cells**

Strong antibacterial activity against *S. aureus* was produced by the administration of the MLC, and a combination of the PPS-BV and MLC in the squid samples. As a consequence, their modes of action towards *S. aureus* were studied through SEM analysis. Untreated *S. aureus* cells exhibited regular and intact cell walls (Fig. 1a). In contrast, the MLC-treated cells of *S. aureus* showed agglutination, adhesion among adjacent cells, and rough surfaces (Fig. 1b). *S. aureus* cells exposed to a combination of the PPS-BV and MLC appeared to be severely damaged, e.g. cell shrinkage, cell adhesion, corrugated surfaces, and with hollow cells that may be indicative of intracellular fluid efflux (Fig. 1c). There was no growth of the *S. aureus* cells treated with both additives whereas the untreated pathogen grew well on the TSA plates (data not shown). This was indicative of bactericidal potential against pathogenic *S. aureus* of the two tested additives.

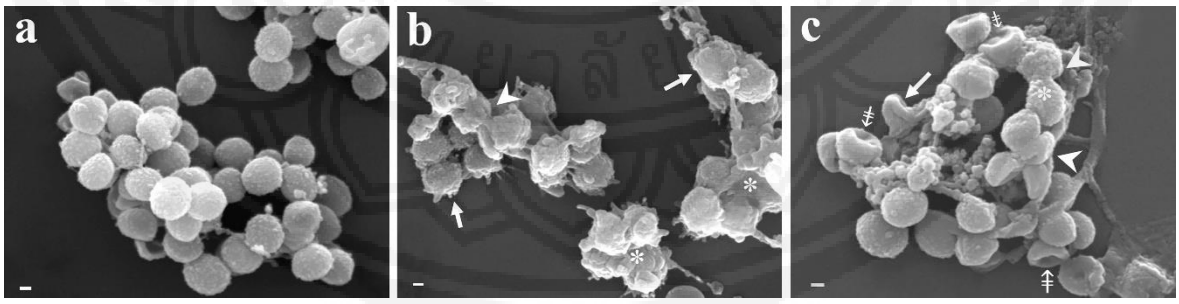


**Figure 1** SEM microphotographs of *S. aureus* treated with the MLC or a combination of the PPS-BV and MLC for 20 hr. a) undamaged cells of the control *S. aureus*, b) agglutination (✻) and adhesion cells (➤) along with corrugated surfaces (↑) of *S. aureus* cells treated with the MLC, and c) *S. aureus* treated with a combination of the PPS-BV and MLC showing shrinkage (↑), cell adhesion (➤) and corrugated surfaces (✻) together with hollow cells (↟). Bar = 200 μm

**Physicochemical properties**

Similar pH values were observed in the control and dried squid samples with *S. aureus* addition and distilled water ranging from 6.27±0.05 to 6.29±0.04, which were significantly higher ($p$<0.05) than that of the dried squid samples with the addition of *S. aureus* and the MLC (5.97±0.14) at the onset of the study. During the 28-day storage at both conditions, the pH values of the squid samples with *S. aureus* and MLC addition were significantly lower ($p$<0.05) than those of the control and the squid samples with water (Table 3).

**Table 3** pH values of dried, seasoned and crushed squids with/without the MLC during storage at different conditions for 28 days

| Storage conditions | Duration (days) | Dried squids without any additives | Dried squids with *S. aureus* and distilled water | Dried squids with *S. aureus* and MLC |
|---|---|---|---|---|
| | 15 min | 6.27±0.05 $^{a,1}$ | 6.29±0.04 $^{a,1}$ | 5.97±0.14 $^{b,1}$ |
| Refrigeration | 7 | 6.26±0.02 $^{a,1}$ | 6.30±0.09 $^{a,1}$ | 6.02±0.02 $^{b,1}$ |
| | 28 | 6.28±0.09 $^{a,1}$ | 6.29±0.09 $^{a,1}$ | 5.74±0.04 $^{b,2}$ |
| Room temperature | 7 | 6.06±0.05 $^{bc,2}$ | 6.12±0.08 $^{ab,2}$ | 6.02±0.13 $^{c,1}$ |
| | 28 | 6.16±0.28 $^{a,12}$ | 6.05±0.05 $^{b,2}$ | 5.61±0.04 $^{c,3}$ |

Means with superscript letters indicate a significant difference ($p$ < 0.05) among treatments. Means with superscript numbers indicate a significant difference ($p$ < 0.05) over time.

Under both storage temperatures for 28 days, the lowest $a_w$ values were observed in the squid samples without any additives. On the contrary, $a_w$ values significantly increased ($p$<0.05) in the dried squids with *S. aureus* and the MLC, compared to other treatments during the 28-day storage period (Table 4).

**Table 4** Water activity ($a_w$) of dried, seasoned and crushed squids with/without the MLC during storage at different conditions for 28 days

| Storage conditions | Duration (days) | Squids without any additives | Squids with *S. aureus* and distilled water | Squids with *S. aureus* and MLC |
|---|---|---|---|---|
| | 15 min | 0.491±0.001 $^{c,2}$ | 0.721±0.003 $^{ab,2}$ | 0.714±0.002 $^{b,3}$ |
| Refrigeration | 7 | 0.474±0.003 $^{c,3}$ | 0.773±0.002 $^{a,1}$ | 0.726±0.002 $^{b,23}$ |
| | 28 | 0.528±0.003 $^{c,1}$ | 0.764±0.012 $^{b,1}$ | 0.851±0.009 $^{a,1}$ |
| Room temperature | 7 | 0.481±0.001 $^{c,3}$ | 0.706±0.003 $^{b,2}$ | 0.748±0.001 $^{a,2}$ |
| | 28 | 0.423±0.005 $^{c,4}$ | 0.487±0.004 $^{b,3}$ | 0.633±0.017 $^{a,4}$ |

Means with superscript letters indicate a significant difference ($p$ < 0.05) among treatments. Means with superscript numbers indicate a significant difference ($p$ < 0.05) over time.

## Discussion

The bacteriological safety of traditional seafood-based products in Thailand is a concerning issue owing to extensive handling during preparation and storage for sale. Little is known about the antibacterial potential against foodborne pathogens of biopreservatives in Thai traditional seafood-based products. One option to control the growth of pathogens is the introduction of bacteriocins as a preventive technique in food systems. In this study, the PPS-BV exhibited significantly low inhibitory activity against *S. aureus* in dried, seasoned, and crushed squid samples during a 28-day storage period at both conditions, even when administrated every

14 days. The result was in contrast to our recent *in vitro* study related to the antimicrobial spectrum of the PPS-BV evaluated using the agar well diffusion method (Butkhot et al., 2019b). The authors observed that the PPS-BV had strong anti-staphylococcal activity against *S. aureus* ATCC 25923 with an inhibition zone of 10.7 mm. This solution also strongly inhibited other foodborne and food-spoilage Gram-positive bacteria, namely *Bacillus cereus* TISTR 687, *B. coagulans* ATCC 12245, *Listeria monocytogenes* ATCC 15313, and *Micrococcus luteus* ATCC 9341 with inhibition zones ranging from 14.2 to 17.8 mm. It is well-known that the intrinsic properties of food (food components, water content, pH, salt, and other additives) and extrinsic determinants (temperature, and storage environment) can influence bacterial sensitivity and preservative action. The weakened anti-staphylococcal potential of the PPS-BV in this study is likely due to its reaction with food components, like proteins and fats, and inactivation by indigenous and/or microbial proteolytic enzymes (Pato et al., 2022).

In the present study, an apparent decrease in *S. aureus* was observed in the MLC-treated squid samples during the 28-day storage period under both conditions. Previous studies demonstrated plant-based substances as good candidates to eliminate pathogenic bacteria in food handling and preparation systems. Soodsawaeng, Butkhot, Boonthai, Vuthiphandchai and Nimrat (2021) reported that the MLC had an effective potential to inhibit the growth of pathogenic *E. coli*, *Salmonella* Typhimurium and *Bacillus cereus* in dried, seasoned, and crushed squid during storage indicated by a substantial reduction in the pathogen. Likewise, the antagonistic action of *Pulicaria inuloides* essential oils (PIEO) against *S. aureus* was demonstrated by Al-Hajj et al. (2017). A solution containing either 0.2 or 0.3 g/100g PIEO markedly reduced the population of experimentally inoculated *S. aureus* below the acceptable limit of less than 2 log CFU/g simultaneously with an extended shelf life of kachlan (*Trachinotus ovatus*) fillets while the untreated fillets contained *S. aureus* in the ranges of 4.2-5.9 log CFU/g during a 12-day storage period. SEM analysis revealed that the MLC had bactericidal activity against *S. aureus* through the destruction of the bacterial cell walls. The results were accord with our recent trial reported by Soodsawaeng et al. (2021). Cell agglutination, adhesion among adjacent cells, rough surfaces, cell shrinkage, and ghost cells with cytoplasmic material completely lost were observed when the MLC were in contact with *E. coli*, *S.* Typhimurium and *B. cereus*. The mode of action of the MLC has not yet been investigated. Lemongrass and hot chili are reported to be sources of numerous compounds with diverse chemical structures that have antibacterial potency against foodborne pathogens. The major components of lemongrass extract/essential oil include a cocktail of mixed terpenes and terpenoids, e.g., citral, neral, isoneral, geranial, isogeranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and elemol (Mukarram et al., 2022) while phenolic compounds and capsaicinoids, particularly capsaicin, dihydrocapsaicin, protocatechuic acid, *p*-coumaric acid, cinnamic acid, and ferulic acid exist predominantly in ethanolic chili extract (Menezes et al., 2022). It is plausible that the inhibitory action is not attributable to the given mechanism of action generated from one active ingredient, but there are synergistically different targets in the cells. It is well known that the mechanism of synergistic interaction among active antimicrobial agents includes sequential inhibition of biochemical reactions, the intervention of various protective enzymes, a combination with active components in the cell wall that participate in the entry of other antimicrobials, and interaction with the plasma membrane (Cava-Roda, Taboada-Rodríguez, López-Gómez, Martínez-Hernández, and Marín-Iniesta, 2021). According to Sikkema, Bont, and Poolman (1995), the destruction of the hydrophobic structure and function of the cell membrane is created by essential oil containing monoterpene

compounds due to their lipophilic characteristics. Leakages of intracellular ions ($K^+$, $PO_4^{3-}$, $S^{2-}$) on the cell membrane of *Cutibacterium acnes* and *Staphylococcus epidermidis* are created simultaneously with the cell wall damage, and morphological changes in biofilm cells caused by a specific interaction of phenolic and terpenoid compounds present in thyme (*Thymus vulgaris*) essential oil (Abdelhamed, Abdeltawab, ElRakaiby, Shamma, and Moneib, 2022). Likewise, Cava-Roda et al. (2021) reported the synergistic activity of vanillin in combination with either clove essential oils or cinnamon bark essential oil against foodborne pathogenic *E. coli* O157:H7 and *Listeria monocytogenes*.

The combined addition of plant-based substances and bacteriocins exhibits great potential as antibacterial compounds in food products. In the present study, a combination of the PPS-BV and MLC was effective to control the growth of *S. aureus* in dried, seasoned, and crushed squid during storage. Turgis, Vu, Dupont, and Lacroix (2012) have reported that the combination of either *Brassica hirta* essential oil plus nisin or *Cinnamomum cassia* essential oil plus bacteriocin MT104 produced by *Enterococcus faecium* had additive effects against *S. aureus*. In accordance with Shahbazi, Shavisi, and Mohebi (2016), the combination of nisin (500 IU/g) and essential oil (0.2%) from *Ziziphora clinopodioides* exhibited the effective potential to inhibit the growth of *S. aureus* in raw beef patties. The combination reduced *S. aureus* populations to undetectable levels together with the extension of shelf-life during 9 days of refrigerated storage. In contrast, viable cells of *S. aureus* were in the ranges of 2.7 – 5 log CFU/g in the untreated patty. Field et al. (2015) have also found that the combined bioengineered derivative nisin V and low concentrations of either carvacrol or trans-cinnamaldehyde could reduce viable cells of *L. monocytogenes* in laboratory media, chocolate milk drink and chicken noodle soup. The present study confirmed that the bactericidal potential against *S. aureus* of the novel combined solution appeared to be caused by cell lysis as evaluated by SEM analysis. Similarly, Soodsawaeng et al. (2021) observed bactericidal effect through the deconstruction of the cell wall and cell lysis due to the presence of pore formation in severely damaged cells, when *E. coli*, *S.* Typhimurium and *B. cereus* were exposed to the MLC. This may be associated with synergy among the active components present in the combined solution. It seems that a fluidifying effect on the cell membrane caused by plant essential oil/extract allows the disintegration of the protective outer membrane, increased membrane permeability, inhibition of enzyme function, and a change in the proton motive force resulting in pore formation and increased sensitivity to bacteriocins (Cava-Roda et al., 2021). The mechanisms of action of the novel combined solution should be studied in the future. Further, in this study, the administration of a novel combination of the PPS-BV and MLC was as effective as the MLC for controlling the growth of *S. aureus* in the dried squid samples during storage. This implies that there is no need to use the combined solution as a biopreservative in dried squid. The application of the MLC is appropriate owing to an obvious inhibitory potential against *S. aureus* in the dried squid and the uncomplicated preparation of the solution.

The present study also showed that the anti-staphylococcal activity of the three tested additives, added every 14 days, was higher than that of a single addition in the squid samples during storage. This may be explained by the volatile characteristics of herb extracts. The concentration of active components present in herb extracts may decrease over time during storage to a level at which the active compositions are unable to inhibit pathogen cells when a single treatment is applied (Abdollahzadeh, Rezaei, & Hosseini, 2014). Similarly, the PPS-BV in the dried squid with a single addition possibly reacted with food components and inactivated during storage, thereby weakening the anti-staphylococcal potential (Younes et al., 2017). This

indicates a requirement for a reliable technology for supplementation of the herb-based substances, the bacterial-derived bacteriocin, and their combination in food products while maintaining their antibacterial activities. Edible film derived from natural products is a promising alternative technique that serves as a carrier and allows a controlled release of antimicrobials over an extended storage period in the food handling and preparation system. Additionally, in the present study, *S. aureus* was absent by day 7 of storage at room temperature in all treated groups and the control. On the contrary, the untreated squid samples contained a high *S. aureus* population (>1,100 MPN/g) throughout the 28-day refrigerated storage period. It is widely known that the success of pathogenic bacteria relies on their ability to survive in a wide range of environmental conditions, e.g. temperature, pH, $a_w$, osmotic stress, and salt concentration as well as their resistance to the physical and chemical properties of foods (Saklani, Lekshmi, Nayak, & Kumar, 2020). Olaimat et al. (2021) examined the survival behavior of *S. aureus* in ready-to-eat vegetable salads stored at different temperatures. They reported a minute reduction of *S. aureus* viability in Arabic salad during refrigerated storage. However, the *S. aureus* population dropped to undetectable levels during 5-day storage at 24°C. Similar results were also observed in methicillin-resistant *S. aureus* (MRSA) in seafood products evaluated by Saklani et al. (2020). MRSA numbers remained constant in artificially inoculated Bombay duck fish (*Harpadon nehereus*) stored in ice for 18 days. In dried Bombay duck fish stored at 30°C, MRSA counts decreased by 0.91 log CFU/g in the inoculated sample. Our results suggest that the survival behavior of *S. aureus* in dried, seasoned, and crushed squid is dependent on storage temperature.

Values of pH and $a_w$ are key physicochemical parameters to indicate food quality. In this study, addition of the MLC resulted in a significant decrease in the pH value of the dried, seasoned, and crushed squid during storage at both temperatures despite the strong inhibitory potency produced. This may be due to the pH value (ca. 3.28) of the MLC itself. Azizkhani and Tooryan (2015) demonstrated the pH values of beef sausages with herb-extract addition during chilled storage were dependent on the types of herb extracts. The pH value of the sausage sample containing rosemary (*Rosmarinus officinalis*) extract was significantly higher than that of the control. Conversely, the sausage with the addition of mint (*Mentha longifolia* L. Hudson) extract showed a lower pH value than the control. The authors postulated that the pH values of both extracts may account for such a phenomenon. However, Weerakkody, Caffin, Dykes, and Turner (2011) have reported no apparent changes in pH values in cooked ready-to-eat vacuum-packaged shrimp treated with a mixture of 5 mg/mL galangal (*Alpinia galanga*) and 10 mg/mL rosemary (*R. officinalis*) extracts during storage at 4°C and 8°C. In the present study, $a_w$ values of the MLC-treated squids significantly increased to a point which is over the allowable limit at 0.6 of ready-to-eat dried squids (Thai Industrial Standards Institute, 2010). $A_w$ describes the energy state of available water that participates in microbial growths, chemical-biochemical reactions, rates of deteriorative reactions, and physiochemical properties of food products. A significant increase in $a_w$ over the acceptable limit may affect the stability, safety quality, sensorial quality, and consumer acceptability of the dried squid (Tapia, Alzamora, & Chirife, 2020). It is important to note that $a_w$ plays a role in the inhibition of growth, but food spoilage and pathogenic bacteria are still viable and capable of reproduction on rehydration in food products, thereby posing a potential risk to public health. S. *aureus* requires generally minimal $a_w$ for growth and enterotoxin production as low as 0.86 and 0.87-0.90 (Tapia et al., 2020). In this study, the addition of the MLC resulted in a marked increase in $a_w$ reaching the level at which *S. aureus* may grow and compromise product safety, especially in the 28-day-refrigerated squids (ca.

0.851). Therefore, despite having application potential to control the growth of *S. aureus* in dried squid, it is imperative to develop an effective alternative strategy for the administration of the MLC without the deteriorative effect on the physiochemical quality of foods. Additional studies related to biochemical assessment, texture profile analysis, and sensory evaluation of the dried squids treated with the MLC should also be established to confirm consumer acceptability.

## Conclusion and Suggestions

The MLC was as effective as a combination of the PPS-BV and MLC for controlling the growth of pathogenic *S. aureus* in dried, seasoned, and crushed squid during storage, especially when they were supplemented every 14 days. SEM analysis demonstrated their bactericidal activities against *S. aureus* through the destruction of the bacterial cell walls observed by severe cell damage along with pore formation on cell membranes. The addition of the MLC resulted in a significant reduction ($p<0.05$) in pH value simultaneously with a significant increase ($p<0.05$) in the $a_w$ value of the dried, seasoned, and crushed squid. Our results suggest that the MLC has a promising potential as a biopreservative for controlling the growth of *S. aureus* causing food poisoning to improve safety quality and extend the shelf life of dried seafood-based products of Thailand.

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