Research Article | Food Agricultural Sciences and Technology (FAST) Vol.9, No.1 pages 66-75

Potential application of immobilized *Bacillus subtilis* (P5-6) as bio-protective culture against *Staphylococcus aureus* in acidic and salted food model

Vishal Chhetri[1], Natthanan Buranavanitvong[2], Sarn Settachaimongkon[2], Nattida Chotechuang[2], Cheunjit Prakitchaiwattana[2,3,*]

[1] *Royal Centre for Disease Control, Department of Public Health, Ministry of Health, Thimphu, Bhutan*

[2] *Department of Food Technology, Faculty of Science, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand*

[3] *The Development of Foods and Food Additive from Innovative Microbial Fermentation Research Group, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok, 10300, Thailand Tel.: (+66) 22185515-6, E-mail: Cheunjit.P@chula.ac.th*

[*] *Cheunjit Prakitchaiwattana: Cheunjit.P@chula.ac.th*

Received: 21st January 2023, **Revised**: 16th February 2023, **Accepted**: 17th February 2023

Abstract - This research aimed to apply bacteriocin producing strain *Bacillus subtilis* (P5-6) as protective culture against *Staphylococcus aureus* in bamboo shoot pickle. Expression of genes associated with bacteriocin activity of the *B. subtilis* (P5-6) was investigated. Only subtilin gene expressions were observed along with housekeeping gene BA-*rpoB* when co-cultured with *S. aureus*. *B. subtilis* (P5-6) protective culture was made in a simple form as dried mango pieces containing 2.5 mg/g salt with viable count at 6.25 log CFU/g and water activity (a_w) at 0.49, respectively. The Protective culture was made by immobilizing cells and culture of P5-6 in freeze-dried mango pieces and drying with two-step drying process. The Minimum Inhibitory Concentration (MIC) of the protective culture was 20 AU/mg and Minimum Bactericidal Concentration (MBC) at 80 AU/mg. When applied as bio-preservative in bamboo shoot pickle, the protective culture significantly (p<0.05) reduced the level of *S. aureus* contamination and had no impact on this food properties. This study demonstrated the potential of P5-6 in this simple protective culture form in controlling the growth of *S. aureus*, therefore it could be a potential for further development as bio-preservative to enhanced safety of food products.

Keywords: Bacteriocin, *Bacillus*, protective culture, gene expression, *S. aureus*

1. Introduction

Staphylococcus aureus is a common pathogenic bacterium responsible for a wide and divergent range of human and also animal's infections, including toxin mediated food borne diseases (FBD). *Staphylococcus aureus* is a huge burden, as *staphylococcal* food poisoning (SFP) agent is present as normal flora of food handlers, producing heat and acid tolerant toxins. It can survive under stress conditions, such as low pH, high salt concentration and low water activity. *Staphylococcus aureus* can also produce enterotoxin under conditions of pH; 4-10, Temperature; 7-48°C, NaCl; 0-20%, a_w; 0.83 → 0.99; anaerobic-aerobic (Hennekinne *et al.*, 2012). Therefore, the efforts in food industry is to prevent such bacterial contaminations. Though many chemicals have bacteriostatic or bacteriocidal effect to pathogenic and spoilage micro-organisms but most of these chemicals are not permitted in foods due to their toxicity. Moreover, consumers demand for chemical free food products and their concern about synthetic chemicals used as preservatives of foods. Thus, the use of bio-preservative is an alternative method to overcome this hurdle. Bio-preservation is the use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and to extend the shelf-life of foods. The application of bio-preservative, such as bacteriocin or bacteriocin like inhibitory substances (BLIS), which are generally recognized as safe (GRAS), mostly produced by Gram positive bacteria, including lactic acid bacteria (LAB) and *Bacillus* spp., with the ability to control food pathogens have gained greater attention to be included as food preservatives. In year, 2019, Chhetri *et al.* (2019) and his research group isolated halophilic strains *B.subtilis* (P5-6) from traditional salted Thai fermented fish and investigated bacteriocin production properties to develop as bio-preservative in cottage cheese model. After application in a cottage cheese model, the protective culture could reduce *S. aureus* and significantly helped prolong freshness of the cheese over 16 days without affecting its physicochemical properties, particularly pH. The protective culture was also evaluated as safety agent. In the next few years, To Truc Anh and Prakitchaiwattana (2021) further developed process for production protective culture of this strain and applied as bio-preservative in fresh meat. It was found that this protective culture could extend the shelf life and reduce *Staphylococcus aureus* on this food model.

In food processing, salt is an important additive and fermentation process that have been traditionally used to preserve food. Salt helps reduce water activity (a_w); hence it is used as a hurdle to avoid spoilage and bacterial growth in foods. Thus, foods with low a_w can self-preserve without thermal process. However, some pathogenic bacteria, such as *Staphylococcus aureus* and *Listeria monocytogenes* can survive high salt concentration also with low pH and cause foodborne diseases. (Farber & Peterkin, 1991; Kadariya *et al.*, 2014). Particularly, *S. aureus* may grow and produce enterotoxins in foods with a_w as low as 0.85 and also grow in up to 25% NaCl and even in acidic food. Thus, foods contained high salt and/or acidic food could still be risk of *Staphylococcus aureus* and its toxin. Since *B. subtilis* (P5-6) is a halophilic bacterium that can survive and grow in salted conditions, the protective culture obtained

from this strain would be potential as bio-control to help reduce the risk of *Staphylococcus aureus* in those salted foods. This study therefore aimed to evaluate efficacy of protective culture of *B. subtilis* (P5-6) as bio-control agent in acidic and salted food model, bamboo shoot pickle.

2. Materials and methods

2.1. Bacterial strains

Bacillus subtilis (P5-6) isolated from Plara (Thai traditional salt fermented fish) obtained from collection of Department of Food Technology, Faculty of Science, Chulalongkorn University. Glycerol stock prepared from the master stock was activated by streaking on Nutrient agar at 37°C for 48 hr. The same generation of glycerol stock was used throughout this study. Three strains of *S. aureus*, pathogenic (ATCC 25923 and DMSc 6538) and food isolated (FT30-7), were used as target strains in testing of inhibitory effect of *Bacillus subtilis* (P5-6).

2.2 Bacteriocin gene expression properties assay and inhibitory effect assay

Bacillus subtilis (P5-6) was grown in Nutrient broth (NB) supplemented with 5% NaCl at 37°C in shaking incubator with speed of 150 rpm for 24h. Cell culture was then subjected to Deoxy ribonucleic acid using commercial kit (GF01-1, Vivantis, Malaysia). Primers of subtilin (spa) and subtilosin (sbo) encoding genes were used to perform Polymerase chain reaction. Gel electrophoresis was followed by running PCR products on 1.5% agarose gel stained with ethidium bromide (Chhetri *et al.*, 2019).

The expression of the gene during cultivation was investigated by rRNA extracted at 6th hour of cultivation in the same condition mentioned above, using commercial kit (GF-1 Total RNA extraction kit, Vivantis, Malaysia). RNA was then converted into complementary DNA (cDNA) using reverse transcriptase reaction. The expression of bacteriocin encoding genes was confirmed along with housekeeping gene (HKG-1389472946 *rpoB*). The PCR products were checked on 1.5% agarose gel electrophoresis (Chhetri *et al.*, 2019). Inhibitory activity of P5-6 culture and cell-free culture on *S. aureus* was tested using spot-on-lawn antagonism assay (Moraes *et al.*, 2010; Shin *et al.*, 2008).

2.3 Protective culture production and inhibitory effect on target strains

Protective culture was prepared in the form of immobilized *Bacillus subtilis* (P5-6) culture in freeze-dried mango pieces. Cell culture was obtained by growing bacterial culture to mid log (OD 0.1 600 nm) in 5% NaCl NB, overnight at 37°C. Cell culture in mid log (10^7-10^8 CFU/ml) nutrient broth was supplemented with 5% NaCl. 5ml of broth and 6g of small freeze-dried mango pieces were mixed. The inoculated pieces of mango were dried with two-step drying process, modified from (Rogers, 1914). The protective culture in this form was stored in sealed aluminum foil punch. The minimal inhibitory concentration/ minimal bactericidal concentration (MIC/ MBC) of bacteriocin available in protective culture prepared and after immobilized in mango pieces were determined using the broth microdilution method. This was to study if any deleterious effect of food matrix and drying process to the efficiency

of bacteriocin produced by (P5-6) strains. The MIC and MBC of the prepared protective culture were estimated following Clinical Laboratory Standard Internationals (CLSI) guidelines. The broth micro dilution method was employed to determine MIC/MBC. of *Bacillus* (P5-6) culture. 50µl of sterile nutrient broth was poured into microtiter well. Then 100µl of protective culture suspension (1:100 w/v) was added to the 1st well and then serially diluting by transferring 50µl from each well till the 11th well. A cocktail was prepared by adding three *S. aureus* strains at a volume ratio of 1:1:1 (0.1 50 µl of mixed *S. aureus* suspension (at 0.1 OD) was added to all well except the control well. The microtiter plate was incubated for 24 hours at 37°C. After 24 hours the plate was read at 600nm and 5-10 µl of cultured broth was cultured on 5% NaCl+NA at 37°C, for 24 hours.

2.4 Test of protective culture in bamboo shoot pickle

Food samples for testing were collected and brought to laboratory from Bhutan, bamboo shoot pickle (Figure. 1 (a)) locally manufactured at Gelephu Bhutan were purchased from point of sale and transported to laboratory for study purpose. The food samples were handled aseptically. Pickles were stored in room temperature until further study.

The samples were prepared in a portion of pickle in bottle of 50 grams each. Prior to application of protective culture, 10g of samples were homogenized using Stomacher (AES Labotorie, France) and diluted with 90 ml of 0.1% peptone water (1:10) for testing total viable count (TVC), total yeast and mould count, *S. aureus* (Compact Dry *Staphylococcus aureus* (XSA) NISSUI), pH and also salt concentration. The first portion was mixed with the protective culture and other was used as control treatment. The activity of protective culture to produce bacteriocin and its activity of was monitored during storage period at room temperature (26-27°C). TPC, *S. aureus* and pH were determined every 7 days, for 4 weeks. Partial organoleptic test, such as color changes, order and consistency were examined were observed physically. The tests were conducted in 3 replications.

2.5 Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons by Tukey's test were performed using IBM-SPSS statistics package version 22 (SPSS Inc., Chicago, IL, USA). A probability at $p<0.05$ was considered statistically significant.

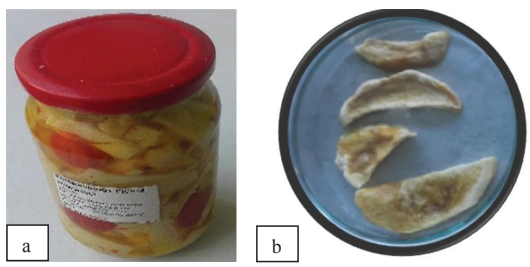

Figure 1. (a) Bamboo shoot pickle (b) Freeze-dried mango piece contained protective culture to be used in the bamboo shoot pickle

3. Results and discussions

3.1 Bacteriocin gene expression and inhibitory effect on *Staphylococcus aureus*

There were some previous reports demonstrated the bacteriocin production of Bacillus strains. Subtilosin and subtilin

produced by B. subtilis and as well as B. amyloliquefaciens, B. licheniformis, B. atrophaeus and other Bacillus strains have been widely demonstrated (Abriouel *et al.*, 2010). In this study, it was therefore investigated for the presence of these genes; subtilin and subtilosin using multiplex PCR protocol and using the primers mention by Klein *et al.* (1993) and Stein (2005). In Figure 2, B. subtilis (P5-6) presented with both of the genes encoding, subtilin and subtilosin (566bp and 876bp respectively). It also exhibited significantly large ($p<0.05$) inhibitory action zone ((Figure 2 (a) and (b)). To confirm the action due to bacteriocin, the bacteriocin encoding gene expression was evaluated along with cocktail S. aureus co-culture. The House keeping Gene (HKG- rpoB) was used as the internal control of the test procedure. The rpoB gene encodes the β-subunit of bacterial RNA polymerase responsible for drug resistance (Halling *et al.*, 1978). It is the second-largest polypeptide in the bacterial cell and codes for 1342 amino acids (Ko *et al.*, 2004). While screening for gene expression of B. subtilis (P5-6) inoculated along with S. aureus co-culture in 5% NaCl nutrient broth, only subtilosin gene expression was observed. This indicated through the presenting of cDNA band (876bp) of this gene on agarose gel (Figure 2 (c)).

Figure 2 (d) showed the co-relation of gene expression with the inhibitory effect of cell free supernatant (CFNS) on cocktail *S. aureus.* CFS collected from the isolates were collected and filtered through 0.45 µm filtrate. Then, 10 µl of CFS was applied on NA (supplemented with 5% NaCl) seeded with cocktail *S. aureus*. The plates were incubated at 37ºC for 24 hours. The result showed that CFS had inhibitory action. Interestingly, this result co-related to the gene expression result on agarose gel electrophoresis obtained from the RNA product (Figure 2 (c)). As found in this study, at 5% NaCl, the gene expression assay was conducted after cultivated the bacterial cell for 24 h when the RNA supposed to be disappeared, the strain *B. subtilis* (P5-6) could still exhibit the gene over expression. This demonstrated the strains might possess genetic property that can over express the gene expression, as presented in Figure 2 (d) with significant inhibitory action.

According to the properties as discussed above, the isolates *B. subtilis* (P5-6) and condition using 5% NaCl was selected to further produce bacteriocin to develop as protective culture for application in acidic and salted food model.

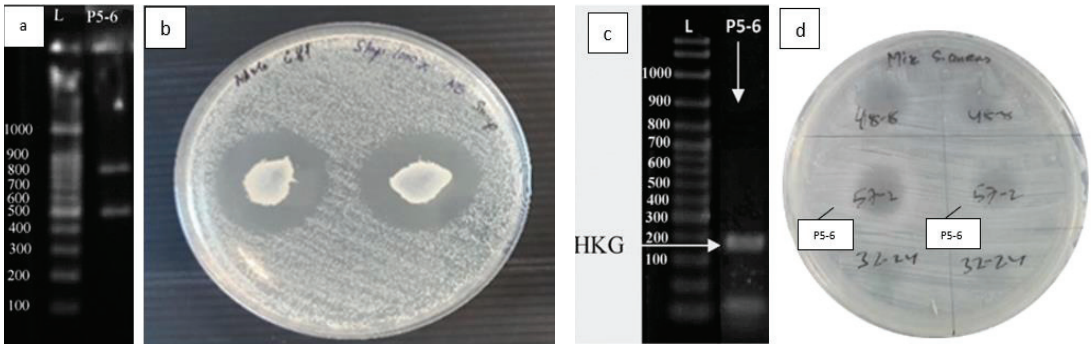

Figure 2. (a) Gene encoding subtilin (566bp)/subtilosin (876bp) of *Bacillus subtilis* (P5-6)
(b) Inhibition zone of (P5-6) on *Staphylococcus aureus*
(c) Gene expression of subtilin (566bp) or subtilosin (876bp) along with housekeeping gene BA-*rpoB* observed in (a) cultures of tested bacterial isolates
(d) ZoI produced by cell free supernatant (CFS), correlating the inhibitory action to gene over expression.

3.2 Protective culture powder and inhibitory properties

Protective starter culture was prepared using freeze-dried mango pieces as matrices (Figure 1 (b)), protectants and also carrier of P5-6 cell and culture to be applied into food. This food grade matrix was selected, as the freeze-dried mango pieces have pores to adsorb the *Bacillus* cells to be fixed and protect during drying process. The use of bacteriocinogenic *Bacillus* strains as starter in food systems could contribute to the competitiveness of the producer strains and to the prevention of food spoilage and pathogenic bacteria. Alternatively, the lateral growth of starter bacteria and production of secondary metabolites could rather enhance its activity (De Vuyst, 2000, Leroy *et al.*, 2003). The amount of protective culture to be applied was calculated from the MBC value and then added ten times more to the food system considering the punitive environment in the food system (De Vuyst, 2000). Thus, properties of protective culture in form of dried mango pieces obtained were therefore investigated. The average total plate count and water activity (a_w) of the final protective culture was found approximately at $1.8x10^6$ CFU/g and a_w 0.49 in freeze-dried mango pieces protective culture, respectively (shown in table 1). The MBC was confirmed again prior to application into the food and its was found to be at 20AU/ml (table 1). The Protective starter culture was kept in the aluminium foil pack and stored at room temperature.

Table 1. Properties of freeze-dried mango pieces contained protective culture

Properties				
Salt concentration	**a_w**	**Viable cell (CFU/g)**	**MIC**	**MBC**
0.25% in 5mg	0.49	$1.8x10^6$	80-160 AU/ml	20 AU/ml

3.3 Mango pieces contained protective culture and bio-control activity in food model

As previously mentioned, protective culture was prepared by direct inoculation of *Bacillus subtilis* (P5-6) culture into freeze-dried mangoes piece. Apart from production of bacteriocin, the starter culture also acts as protective culture, by eliminating the growth of other unwanted bacteria (Woraprayote *et al.*, 2016). In this part, the protective culture in form of *B. subtilis* immobilized in mango pieces was added to the food samples. One gram (P5-6) protective culture added to 90g of samples (1:10 ratio). The protective was added 10 times the MBC value to withstand and adapt the food matrix.

Food model used in this study; bamboo shoot pickle is a product that has gained popularity among the travelers in the Bhutan. It is easily available along the road side vendor, kept for selling by the local villagers. Microbiological analysis, initial total plate count, and yeast and mold count was performed, along with some general properties, including pH, total titratable acidity (TTA) and salt content. Safety evaluation was done for *S. aureus* contamination. Table 2 below shows the complete report for food sample analysis. Pickle is acidic food. Generally, in foods having low pH, only few microorganism, including *S. aureus* can survive the low pH and other pathogenic bacteria's supposed to be inhibited (Radford and Board, 1993). However, in the sample tested, the presence of *Staphylococcus aureus* in food samples were still found to >log 3 CFU/g. The fate of the home-made products usually have the high contamination with *S. aureus* due to low hygienic practice, and also because the food are made by using bare hands, thereby transferring microbes from hands into the food. Therefore, to prevent the contamination of food due to *S. aureus* is important. These selected food samples, despite being acidic and salted food, *S. aureus* were still presented. So, a need for good manufacturing practice and an alternative to overcome *S. aureus* contamination should be taken into consideration.

Table 2. Initial report of bamboo shoot pickle analysis

TPC CFU/ml[A]	Yeast and Mold count CFU/ml[A]	*S. aureus* count CF U/ml[A]	pH[A]	NaCl Conc. mg/g %[A]	TTA %[A]
$3.5x10^4$	<100	$1.6x10^3$	4.0	0.85	2.42

The mango pieces protective culture was added to bamboo shoot pickle and incubated at room temperature (26-28°C) for one month (4 weeks). Total plate count, *S. aureus*, pH and partial organoleptic testing was done every 7 days. There was no obvious physical changes between the samples and the control until the 4th week, thereby indicating that, the addition of *Bacillus* starter didn't produce too much of hydrogen sulphite or otherwise would have changed the color to dark brown. The pH value between the samples and the control recorded at 4th week were 4.0 (Figure 3(a)). The TPC ranged from log 3 and log 4. Initially the amount of *S. aureus* in pickle was detected at 3 log and the count reduced to <100 CFU/ml after addition of protective culture after week 2. After the 2th week, increase of TPC was observed

(Figure 3(b)). This could be due to an activity of lactic acid bacteria surviving in this food model. This indicated that the (P5-6) protective culture had no inhibitory effect of LAB associated to product fermentation. On the other hand, the initial viable *S. aureus* count was recorded at log 3. The *S. aureus* count remained constant in the control but the viable count of *S. aureus* in sample added *Bacillus* protective culture, the count was reduced by more than 2 log CFU as shown in Figure 4, this indicated the inhibitory effect of the protective culture on control *S. aureus* in the bamboo shoot pickle.

It can be concluded that the addition of *B. subtilis* in the simple form of protective culture as proposed in this study could significantly reduce contaminating *S. aureus* in food system. This could be related to the production of bacteriocin by *B. subtilis*, thereby indicating the success of using *B. subtilis* (P5-6) as a bio-preservative in food system.

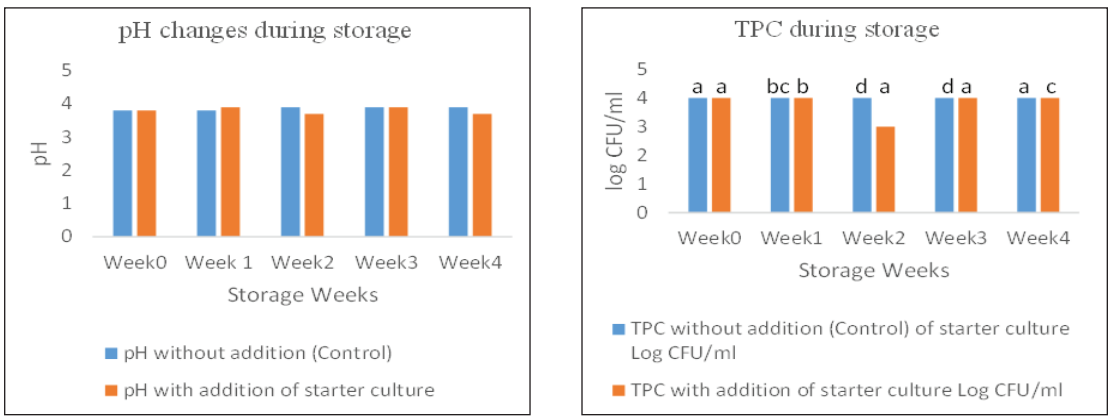

Figure 3. Effect of addition of Protective starter culture in bamboo shoot pickle
(a) Change in pH over the storage weeks at $p<0.05$-
(b) Change in TPC over the storage weeks at $p<0.05$

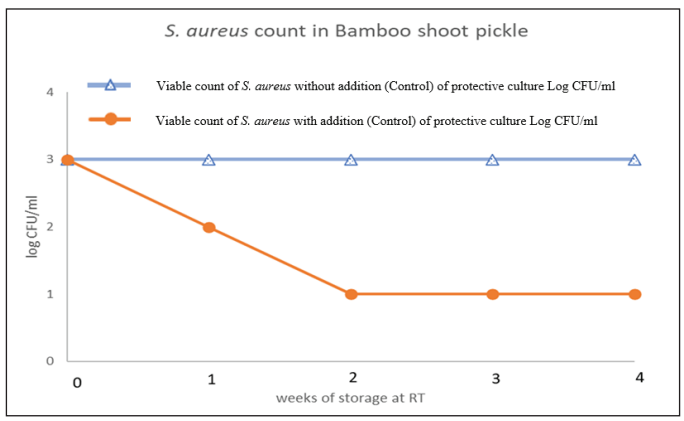

Figure 4. *S. aureus* count in bamboo shoot pickle sample, stored at RT for 4 weeks.

4. Conclusions

These results suggested that the halophile *B. subtilis* (P5-6) could probably produce subtilosin. This confirmed by the gene expression along with inhibitory action against *S. aureus*. Protective culture was prepared by immobilized *Bacillus subtilis* (P5-6) culture in freeze-dried mango piece and dried with two-step drying. This protective culture form displayed inhibitory effect on cocktail-culture of *S. aureus*. Application of the P5-6 protective culture in bamboo shoot pickle displayed the reduction of *S. aureus* from 3 log CFU/g to lower 1 log CFU/g with no impact on the other properties of the pickle. This demonstrates the success of using *B. subtilis* starter as a bio-preservative in food system.

5. Acknowledgements

The authors are grateful to the Research Program for Development of Small and Medium Enterprise Researchers from National Research Council of Thailand, the Thailand Research Fund and the Thailand Research Organizations Network (TRON) years 2018 and 2020 and the Development of Foods and Food Additive from Innovative Microbial Fermentation Research Group, Chulalongkorn University in providing funds for this research.

6. References

Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2010). Diversity and applications of Bacillus bacteriocins. *FEMS Microbiology Reviews, 35*, 201-232.

Chhetri, V., Prakitchaiwattana, C., & Settachaimongkon, S. (2019). A potential protective culture; halophilic Bacillus isolates with bacteriocin encoding gene against Staphylococcus aureus in salt added foods. *Food Control, 104*, 292-299.

De Vuyst, L. (2000). Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology, 38*, 105-112.

Farber, J., & Peterkin, P. (1991). Listeria monocytogenes, a food-borne pathogen. *Microbiological Reviews, 55*, 476-511.

Halling, S. M., Burtis, K. C. & Doi, R. H. (1978). β′ subunit of bacterial RNA polymerase is responsible for streptolydigin resistance in Bacillus subtilis. *Nature, 272*, 837-839.

Hennekinne, J.A., De-Buyser, M.L., & Dragacci, S. (2012). Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiology Reviews, 36*(4), 815-836.

Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International,* 2014.

Klein, C., Kaletta, C., & Entian, K. (1993). Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/ response regulator system. *Applied and Environmental Microbiology, 59*, 296-303.

Ko, K. S., Kim, J.-W., Kim, J.-M., Kim, W., Chung, S.-I., Kim, I. J. & Kook, Y.-H. (2004). Population structure of the Bacillus cereus group as determined by sequence analysis of six housekeeping genes and the plcR gene. *Infection and Immunity, 72*, 5253-5261.

Leroy, F., Moreno, M. F. & De Vuyst, L. (2003). Enterococcus faecium RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *International Journal of Food Microbiology, 88*, 235-240.

Moraes, P. M., Perin, L. M., Ortolani, M. B. T., Yamazi, A. K., Vicosa, G. N., & Nero, L. A. (2010). Protocols for the isolation and detection of lactic acid bacteria with bacteriocinogenic potential. *LWT-Food Science and Technology, 43***,** 1320-1324.

Rogers L. A. 1914; The preparation of dried cultures. *J. infect. Dis, 14*, 100.

Shin, M., Han, S., Ryu, J., Kim, K., & Lee, W. (2008). Isolation and partial characterization of a bacteriocin produced by Pediococcus pentosaceus K23-2 isolated from Kimchi. *Journal of Applied Microbiology*, *105*, 331-339.

Stein, T. (2005). Bacillus subtilis antibiotics: Structures, syntheses and specific functions. *Molecular Microbiology, 56*, 845-857.

To Truc Anh, H., Chhetri, V., Settachaimongkon, S. and Prakitchaiwattana, C. (2021). Stress tolerance-Bacillus with a wide spectrum bacteriocin as an alternative approach for food bio-protective culture production. *Food Control, 133*, A, 108598.

Woraprayote, W., Malila, Y., Sorapukdee, S., Swetwiwathana, A., Benjakul, S., & Visessanguan, W. (2016). Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Science, 120*, 118-132.