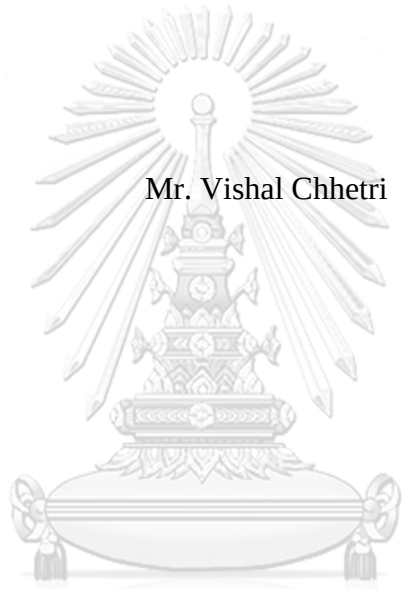
HALOPHILIC BACTERIA FROM SALTY FERMENTED FOODS AND ITS BACTERIOCIN ENCODING GENE EXPRESSION TO USE AS BIOCONTROL AGENT AGAINST *STAPHYLOCOCCUS AUREUS* IN SALT ADDED FOODS

Mr. Vishal Chhetri



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food Science and Technology
Department of Food Technology
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แบคทีเรียซอบเกลือจากอาหารหมักเค็มและการแสดงออกของยีนที่เป็นรหัสของการสร้างแบคทีริโอซินเพื่อใช้เป็นสารควบคุมทางชีวภาพในการต่อต้าน *Staphylococcus aureus* ในอาหารที่เติมเกลือ. อาจารย์ที่ปรึกษาหลัก รองศาสตราจารย์ ดร.ชื่นจิต ประกิตชัยวัฒนา อาจารย์ที่ปรึกษาร่วม อาจารย์ ดร.ศานต์ เศรษฐชัยมงคล

นายวิสเซล เซ็ทตรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	HALOPHILIC BACTERIA FROM SALTY FERMENTED FOODS AND ITS BACTERIOCIN ENCODING GENE EXPRESSION TO USE AS BIOCONTROL AGENT AGAINST *STAPHYLOCOCCUS AUREUS* IN SALT ADDED FOODS
By	Mr. Vishal Chhetri
Field of Study	Food Science and Technology
Thesis Advisor	Associate Professor Cheunjit Prakitchaiwattana
Thesis Co-Advisor	Sarn Settachaimongkon

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich)

THESIS COMMITTEE

.....Chairman
(Associate Professor Sumate Tantratian)

.....Thesis Advisor
(Associate Professor Cheunjit Prakitchaiwattana)

.....Thesis Co-Advisor
(Sarn Settachaimongkon)

.....Examiner
(Associate Professor Chanprapa Imjongjirak)

.....Examiner
(Nattida Chotechuang)

.....External Examiner
(Assistant Professor Romanee Sanguandeekul)

วิสเชลเชีทตรี : แบคทีเรียชอบเกลือจากอาหารหมักเค็มและการแสดงออกของยีนที่เป็นรหัสของการสร้างแบคทีริโอซินเพื่อใช้เป็นสารควบคุมทางชีวภาพในการต่อต้าน *Staphylococcus aureus* ในอาหารที่เติมเกลือ. อาจารย์ที่ปรึกษาหลัก รองศาสตราจารย์ ดร.ชื่นจิต ประกิตชัยวัฒนา อาจารย์ที่ปรึกษาร่วม อาจารย์ ดร.ศานต์ เศรษฐชัยมงคล (HALOPHILIC BACTERIA FROM SALTY FERMENTED FOODS AND ITS BACTERIOCIN ENCODING GENE EXPRESSION TO USE AS BIOCONTROL AGENT AGAINST *STAPHYLOCOCCUS AUREUS* IN SALT ADDED FOODS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ชื่นจิต ประกิตชัยวัฒนา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศานต์ เศรษฐชัยมงคล, 120 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อคัดแยกเชื้อแบคทีเรียชอบเกลือที่สร้างสารยับยั้งการเจริญของจุลินทรีย์จากผลิตภัณฑ์อาหารหมักพื้นบ้าน ได้แก่ ปลาร้าและซีอิ้ว เพื่อประยุกต์ใช้เป็นสารชีวภาพควบคุมจุลินทรีย์ในผลิตภัณฑ์อาหารที่มีเกลือ โดยการคัดแยกเชื้อ วิเคราะห์ระบุสายพันธุ์ และศึกษาคุณลักษณะเฉพาะของแบคทีเรียที่มีฤทธิ์ในการยับยั้ง *Staphylococcus aureus* สายพันธุ์ต่างๆ แล้วพัฒนาเป็นกล้าเชื้อแบบโพรเทคทีฟผลิตภัณฑ์อาหารต้นแบบ การศึกษาความหลากหลายของประชากรจุลินทรีย์ในปลาร้าและซีอิ้วใช้วิธีเพาะเลี้ยงบนอาหารเลี้ยงเชื้อควบคู่กับการวิเคราะห์ทางอณูชีววิทยา ด้วยเทคนิค Reverse Transcriptase PCR-DGGE (Rev-T PCR-DGGE) และการวิเคราะห์ลำดับเบสบน 16s rDNA ผลที่ได้จากการวิเคราะห์บนอาหารเลี้ยงเชื้อพบว่าแบคทีเรียไอโซเลทส่วนใหญ่ที่พบในตัวอย่างอาหารทั้งสองชนิดคือ *Bacillus* spp. โดยมี *B. subtilis* เป็นสายพันธุ์หลัก (ร้อยละ 41) ซึ่งแตกต่างจากผลการวิเคราะห์ด้วย Rev-T PCR-DGGE ที่พบว่า *Halanaerobium* spp. เป็นกลุ่มแบคทีเรียหลักในปลาร้า และ พบ *Staphylococcus gallinarum* เป็นแบคทีเรียหลักในซีอิ้ว ในงานวิจัยนี้สามารถคัดแยกเชื้อได้เบื้องต้นทั้งหมดรวม 214 ไอโซเลต พบว่ามี 37 ไอโซเลท (ร้อยละ 29.8) เป็นแบคทีเรียในสกุล *Bacillus* spp. 35 ไอโซเลท และแบคทีเรียกลุ่มอื่นอีก 2 ไอโซเลท ที่สามารถยับยั้ง *S. aureus* ทั้ง 3 สายพันธุ์ ได้อย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่นร้อยละ 95 เมื่อทดสอบด้วยวิธี spot-on-the-lawn จากนั้นนำ *Bacillus* ไอโซเลทที่ให้ผลบวกไปทดสอบการแสดงออกของยีน subtilin-*spa* และ subtilosin-*sbo* ที่เกี่ยวข้องกับการสร้างสารแบคเทอริโอซิน พบว่าเชื้อ *B. subtilis* และ *B. licheniformis* มีการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างแบคเทอริโอซินในระดับสูง (over expression) เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีเกลือความเข้มข้นร้อยละ 5 และเมื่อเลี้ยงในรูปแบบเชื้อผสม (co-cultivation) ร่วมกับ *S. aureus* จากนั้นคัดเลือก *Bacillus* ไอโซเลท ที่ผ่านการประเมินด้านความปลอดภัยไปพัฒนาเป็นกล้าเชื้อสำหรับทดสอบในอาหารต้นแบบ โดยเชื้อดังกล่าวให้ผลการทดสอบค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถฆ่าเชื้อแบคทีเรีย (minimum bactericidal concentration; MBC) และค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย (minimum inhibitory concentration; MIC) ที่ระดับ 20 AU/ml และ 80 AU/ml ตามลำดับ และเมื่อนำเชื้อดังกล่าวไปทดสอบในอาหารต้นแบบ 2 ชนิด ได้แก่ เนยแข็งสดชนิด cottage และหน่อไม้ดอง พบว่าสามารถลดระดับการปนเปื้อน *S. aureus* และยังช่วยยืดอายุการเก็บนานอย่างมีนัยสำคัญ งานวิจัยนี้แสดงให้เห็นถึงศักยภาพของแบคทีเรียในสกุล *Bacillus* spp. สายพันธุ์ที่คัดแยกได้จากแหล่งอาหารหมักตามธรรมชาติ เพื่อใช้สำหรับควบคุมการปนเปื้อนของ *S. aureus* และแนวทางการประยุกต์ใช้ในรูปแบบวัตถุกันเสียชีวภาพ (bio-preservative) เพื่อเสนอเป็นทางเลือกใหม่สำหรับอุตสาหกรรมอาหาร

ภาควิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีทางอาหาร	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

5872635523 : MAJOR FOOD SCIENCE AND TECHNOLOGY
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VISHAL CHHETRI: HALOPHILIC BACTERIA FROM SALTY FERMENTED FOODS AND ITS BACTERIOCIN ENCODING GENE EXPRESSION TO USE AS BIOCONTROL AGENT AGAINST *STAPHYLOCOCCUS AUREUS* IN SALT ADDED FOODS. ADVISOR: ASSOC. PROF. DR CHEUNJIT PRAKITCHAIWATTANA, CO-ADVISOR: DR SARN SETTACHAIMONGKON, 120 pp.

This research was aimed to isolate, select and apply bacteriocin producing halophilic bacteria from salty fermented foods, Plara (Thai traditional salty fermented fish) and soya sauce as bio-control agent in foods. The isolates having inhibitory activity against different strains of *Staphylococcus aureus* were selected, identified and characterized prior to application as protective starter in food models. Bacterial communities of Plara and soya sauce samples were studied by two methods, a cultural dependent and cultural independent method (Reverse Transcriptase PCR DGGE (Rev-T PCR-DGGE)), and subsequently sequenced by 16s rDNA analysis. *Halanaerobium* spp. in Plara and *Staphylococcus gallinarum* in soya sauce were the main population detected by the Rev-T PCR-DGGE, while *Bacillus* spp. with the predominance of *B. subtilis* (41%) was the bacterial isolates detected by cultural plating method. Among 124 isolates from soya sauce and Plara, 37 (29.8%) isolates (35 *Bacillus* strains and 2 other groups of bacteria) exhibited inhibitory effect against the three different indicator *Staphylococcus aureus* strains. The inhibitory action was tested by deferred antagonism, spot-on-the-lawn method. The *Bacillus* isolates displayed different inhibitory pattern on the indicator strains, significantly different at $p<0.05$. The *Bacillus* isolates with positive inhibitory action was further investigated for the gene encoding bacteriocin production (subtilin-*spa*/ subtilosin-*sbo*). Furthermore, bacteriocin gene expression was studied, and found that two isolates of *Bacillus* (*B. subtilis* and *B. licheniformis*) over expressed bacteriocin gene in 5% NaCl and co-culture with cocktail *S. aureus*. The gene expression *Bacillus subtilis* isolate was selected for developing to protective culture, after subjected to safety evaluation. Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of the selected strain were found at 20AU/ml and 80AU/ml, respectively. When application as protective cultures in two food models (cottage cheese and bamboo shoot pickle), the culture significantly reduced 2-3log cycle of *S. aureus* contamination. This study demonstrated a potential of *Bacillus* spp. in controlling the growth of *S. aureus*, therefore it could be a potential in further development as protective culture in food industries.

Department:	Food Technology	Student's Signature .....
Field of Study:	Food Science and Technology	Advisor's Signature .....
Academic Year:	2017	Co-Advisor's Signature .....

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CHAPTER 1

INTRODUCTION

Staphylococcus aureus is a common pathogenic bacteria causing many 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, with the ability for production of heat and acid tolerant toxins. *Staphylococcus aureus* can survive stress conditions, such as, relatively low water activity, high salt (Martirani et al.) concentration and low pH. Therefore, the concerns in food industry is to prevent such bacterial contaminations.

Though many chemicals have bacteriostatic or bactericidal effect to pathogenic and spoilage micro-organisms but most of these chemicals are not permitted in foods due to their toxicity and clean label demand from the food safety authorities. 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 attractive alternative method to overcome this hurdle. Bio-preservation is the use of safe microorganisms and/or their antimicrobial peptides to improve microbiological food safety and to prolong 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.

Bacillus represents a genus for the identification of bacteriocin because it includes many industrial species. It is also considered to be the second most important bacteriocin producer following LAB. *Bacillus* produce bacteriocin that has wide and broad range of inhibitory activities, on the other hand, LAB bacteriocin are usually narrow antimicrobial spectrum. Few well characterized lantibiotics from *Bacillus* includes, subtilosin, subtilin, amylolysin and amylocyclicin. This group of bacteriocin produce inhibitory action against various species of Gram positive bacteria, including *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*, and Gram negative bacteria, including *Escheria coli* and *Pseudomonas aeruginosa*. The

commonly isolated *Bacillus* from fermented foods; such as fermented fish, and soya sauce and known for production of many natural antimicrobial peptides includes *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus atrophaeus*

Nisin, which is produced by *Lactococcus lactis* discovered in 1928, is the only bacteriocin currently being permitted by U.S. FDA to be applied into food system. But the use of nisin is limited nowadays as some strain of *Listeria* already started to become resistant. Though, several bacteriocin produced by LAB and *Bacillus* have been studied and categorized based on their molecular weight/size, mode of action and structure, but yet to be approved for application in commercial food systems. Therefore, the need for more research to find safe and active compounds that has broad antibacterial property has become more important than ever and to be approved as food grade.

The aim of this study was to isolate bacteriocin or BLIS producing halophilic bacteria from Plara and soya sauce, to be developed as protective culture, to be used in salt added foods, being able to control *S. aureus* contamination. Safe food is every individual right.

1.1 Objectives:

i. To isolate Halophilic bacteria from salty fermented foods and study the bacterial community.
ii. To evaluate halophilic isolates producing bacteriocin, gene encoding and gene expression having inhibitory effect on *Staphylococcus aureus.*
iii. To evaluate efficacy of bacteriocin producing halophilic bacteria as bio-control agent and application in salt added food.

CHAPTER 2

LITERATURE REVIEW

2.1 Preservatives and Bio-preservation

Preservatives are compounds that kill or control the spoilage of food due to the activity of microorganisms and help prolong the shelf-life by decreasing or limiting the rate of deterioration (Brul and Coote, 1999). Food preservatives can be either chemical/artificial preservative or natural/bio-preservatives. Many chemicals have bacteriostatic or with bactericidal effect to micro-organisms but most of these chemical are not permitted in foods due to their toxicity; chemical preservatives include chemicals with antimicrobial; bactericidal or bacteriostatic properties, such as benzoic acid, sodium nitrate, calcium propionate, nitrite and sulfites (sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.). As mentioned in FAO, 1991, chemical food preservatives are defined as, those chemical substances which are added in small volume (up to 0.2%) and which do not change or modify the organoleptic and physical-chemical properties of the foods at all or very minor change. On the other hand, the use of safe, food grade microorganisms and/or their peptides to improve microbiological safety and prolong the shelf life of foods is termed as bio-preservation (De Martinis et al., 2001). Fermentation is a common and oldest forms of food bio-preservation that converts sugar to acids, gases, or alcohol. It is a process that depends on the growth of microorganisms (yeast or bacteria) in foods, added either by allochthnous or autochthnous method.

2.1.1 Fermentation

The science of fermentation is known as zymology. French microbiologist; Louis Pasteur (1822 – 1895) is remembered for his insights into microbial fermentation (Ross et al., 2002). There are many types of fermentation, including lactic acid fermentation and alcoholic fermentation. Alcoholic fermentation generally means production of ethanol (CH_3CH_2OH) and lactic acid fermentation can be either homo or hetero fermentative yielding lactic acid as an end product. Both the biochemical and microbiological studies on fermentation have been conducted. Preservation of food as

a result of fermentation has been a successful means of prolonging the shelf-life of foods, since the ancient times. This is not only for the preservative purpose, but also to improve nutrients and flavor. Several microorganisms are involved in during fermented food development and usually LAB is the most dominant and desirable microorganism (Rusmana et al., 2013), apart from the other groups of microbes. Addition of salts (sodium chloride) during fermentation process is common practice to avoid the growth of pathogenic organism, referred as hurdle effect. But there are microbes that grow in elevated saline environment and these group of bacteria are referred as halophile bacteria. The common halophile bacteria include, LAB, *Pediococcus halophilus*, and *Bacillus* spp. and yeast *Zygosaccharomyes rouxii*, also isolated from salty fermented foods. Among the many products that involve salty fermentation, fish fermentation (Plara/Plasom), soya sauce, vegetables in brine are popular among South Asian population.

2.1.2 Plara

Plara is a fermented traditional fish product, consumed among Mekong region (including Thailand), made by mixing freshwater fish with roasted rice and high salt, then leaving this mixtures at room temperature for 6-12 months (Yachai et al., 2008). Fish is highly perishable food due to the free amino acids and nitrogen bases (Gómez-Sala et al., 2016), then fish fermentation is practiced. Plara is commonly consumed as seasoning in Thailand and various South East Asian countries.

2.1.3 Soya sauce

Traditionally fermented soy product includes soybean paste, soy sauce, tempeh and also soymilk products (Kim et al., 2010). Soya sauce is a two stage fermentation processed product of soya bean, along with roasted wheat which also involves mold activities. The first fermentation process involves a yeast solid-state fermentation of plant materials, then followed by a brine fermentation.

2.2 Food borne disease

Food borne illness or diseases (FBD) include a wide-ranging types of illnesses and are a growing public health problem worldwide, apart from other non-communicable and infectious disease. The food and waterborne diseases are the results of consumption of contaminated foodstuffs and drinking water being contaminated with microorganisms or chemicals. Foodborne diarrheal diseases are a problem for every country, mostly in the developing countries of the world. Diarrhea is the acute and most often symptom associated with foodborne illness, but other serious consequences include kidney and liver failure, brain and neural disorders, reactive arthritis, cancer and death if not treated in time (Lindsay, 1997, Batz et al., 2013). The contamination of food may occur at any stage during the process from food production, preparation to consumption ("farm to fork"). The contaminants may be present in the environment, including pollution of water, soil or air or human. FBD are defined by the World Health Organization (WHO) as diseases caused by the consumption of contaminated food or water. The occurrence of two or more similar illnesses resulting from the ingestion of contaminated food or water is termed as foodborne disease outbreak (Bennett et al., 2013).

2.2.1 Types of food borne diseases

Mostly the food borne illness are of acute onset and causes irritation of gastrointestinal track (GI) due to ingestion of harmful bacteria, parasites, viruses, or chemicals along with the foods. The term foodborne diseases, food-borne infections including foodborne intoxications and toxico-infection, covers illnesses acquired through consumption of contaminated food with micro-organism, and are also frequently referred to as food poisoning (CDC, 2009). There are three types of bacterial food borne diseases; i) Food borne infection is caused by the viable or living cells or spores and cause invasion of intestinal mucosa or other systemic organ (liver/muscle). The common bacteria causing food borne infection includes *Shigella*, *Salmonella*, *E. coli*, and *Vibrio* spp. ii) Food borne intoxication is caused by ingestion of preformed toxins and also known as microbial toxin effects (Zottola and Smith, 1990). The common intoxication is caused by *Staphylococcus aureus* and *Clostridium botulinum*.

iii) The third kind of food borne illness is toxico-infection, caused due to ingestion of bacterial spores and enterotoxin produced, such as *Clostridium perfringens* and *Bacillus cereus.*

2.3 *Staphylococcus aureus*

Staphylococcus aureus is a common pathogen often related with food poisoning outbreaks causing a violent or projectile vomiting due to intoxication through the ingestion of enterotoxins pre-formed in foods (Honório et al., 2015). The first case of isolation of *S. aureus* from food poisoning dates back as early as 1884. *Staphylococcus aureus* is a Gram positive bacteria that can survive harsh conditions like high salt and low pH, as mentioned below in the table 2.1 (Charlier et al., 2009). It forms a grape-like clusters as the daughter cells remain attached to each other during binary fission. It is commonly found as the commensal of skins and upper respiratory tract of human and animals (Kluytmans and Wertheim, 2005). Therefore, it is often referred to *S. aureus* as opportunistic pathogen and mostly associated with hospital acquired infection (HAI)/nosocomial infection (Gaynes et al., 1996), apart from causing food poisoning. Moreover, *S. aureus* is also associated with increase in drug resistance, including methicillin resistance *S. aureus* (MRSA) and the strain is also isolated from many food products (Pesavento et al., 2007, Waters et al., 2011).

Table 2.1 Environmental condition allowing *S. aureus* growth and enterotoxin production

	Bacterial growth		Enterotoxin production	
Conditions	Optimum	Range	Optimum	Range
Water activity (a_w)	>0.99	0.83-0.99	0.98	0.87->0.99
pH	6-7	4.0-10	7-8	4.5-9.6
Temperature	37°C	7-47°C	40-45	10-48
NaCl	0-4%	0-20%	-	-

2.3.1 Staphylococcal enterotoxin

Staphylococcus aureus produces numerous types of toxins, such as staphylococcal enterotoxin SE (exo-proteins) that contribute to pathogen's ability to colonize and cause disease in mammalian hosts. Nearly all strains secrete a group of enzymes and toxins which includes hemolysins (alpha, beta, and gamma), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth (Dinges et al., 2000).

Staphylococcus aureus enterotoxins (SE) is produced throughout the logarithmic phase of growth or during the transition from exponential to stationary phase. They are active and have efficacy in high nanogram to low microgram gram quantities (Argudín et al., 2010) and are resistant to conditions (heat treatment and low pH) that easily destroy the bacteria cells that produce them. Moreover, they are not degraded by proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion (Bergdoll, 1983). There are several different types of SE; enterotoxin A is predominantly associated with staphylococcal food poisoning and enterotoxins B, D, E, H, G and I, are occasionally associated with staphylococcal food poisoning (Argudín et al., 2010, Evenson et al., 1988).

2.3.2 Mode of action

Important efforts have been made to identify specific amino acids and domains within SEs which may be important for emesis or non-emetic. Like toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxin L and Q are non-emetic, while staphylococcal like enterotoxins *I* displays slightly emetic activity (Pinchuk et al., 2010). It has been suggested that staphylococcal enterotoxins stimulate the vagus nerve in the abdominal viscera, which transmits the signal to the vomiting center in the brain, as shown in the figure 2.1. In addition, staphylococcal enterotoxins are able to penetrate the gut lining and activate local systemic immune response. Release of inflammatory mediators, including histamine, leukotrienes and neuroenteric peptide substance causing vomiting. The diarrhea sometimes associated with SEs intoxication may be due

to the inhibition of water and electrolyte reabsorption in the small intestine (Argudín et al., 2010, Sugiyama and Hayama, 1965).

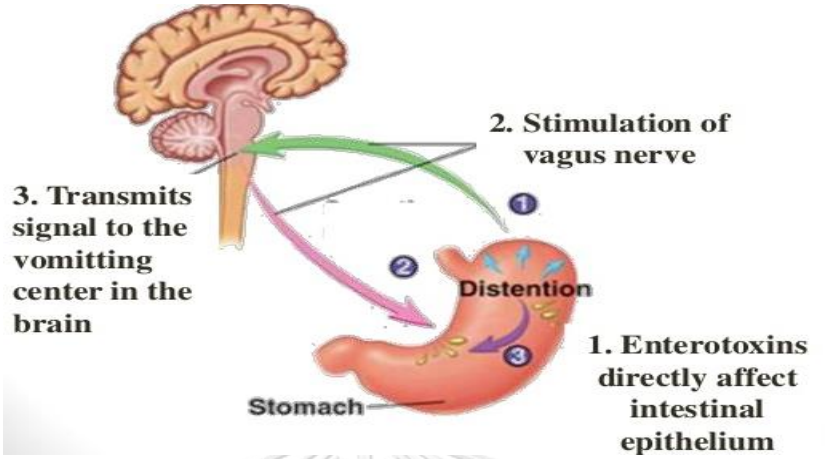


Figure 2.1 Mechanism of Staphylococcal enterotoxin activating vomiting center

2.3.3 Staphylococcal food poisoning

Staphylococcal food poisoning illness is an intoxication that results from the ingestion of foods containing adequate amounts of preformed enterotoxins. Symptoms of SPF include projectile vomiting (violent/rapid vomiting), nausea, and abdominal cramp associated with or without diarrhea. It has short incubation period 30 minutes to 2 hours. The condition is normally not severe and resolves within 24-48 hours. But sometimes, unusually it can be severe in case of infants, immune compromised people and aged population often requiring hospitalization.

The clinical diagnosis of SPF is generally complete by conducting following laboratory investigations (Kérouanton et al., 2007):

i. The retrieval of live *S. aureus* (>log 5) either from vomits of the patients or from food remnants, if available for testing.
ii. The detection of presence of staphylococcal enterotoxins in food remnants.

Foods handlers are usually the source of food contamination with *S. aureus*, as it is mentioned to be found as common commensel of skin (Kluytmans and Wertheim, 2005). Unlike other bacteria *S. aureus* does not survive and compete well with the other microbes present in the system, rather the contamination is associated with post production, such as improper handling of cooked or processed foods, temperature abuse and followed by storage under condition that allow growth of *S. aureus* and favorable for the production of enterotoxin(s) (Argudín et al., 2010). Though *S. aureus* are sensitive to heat treatment, it can easily cause cross contamination as almost 40 % of the people are carriers (Le Loir et al., 2003) and moreover, its ability to survive low pH and high salt concentration adds to pathogenicity. So, this characteristic make *S. aureus* grow in various food products, including fermented, acidic or salt containing foods.

2.3.4 Detection and identification

Various types of methods are available for detection of *S. aureus* contamination in foods, and the use of conventional culture method with isolation and biochemical identification is laborious and time consuming. Therefore, rapid and convenient test methods with high sensitivity and specificity, using compact dry petri plate is used (Kodaka et al., 2005). Compact and dry form of petri plate is a rapid and sensitive method kit for determining microbe aerobic colony counts or any specific bacteria or yeast in foods. Apparently not much variations were detected between the Compact petri plate method and the standard pour plate method (AOAC Official Method) for the detection of anaerobic microorganisms (Kodaka et al., 2005). Compact Dry method allow easy identification and quantification of bacteria.

2.3.5 Early Detection of food borne disease outbreaks

Food borne disease are usually associated with outbreaks; a foodborne disease outbreak is defined as the occurrence of two or more similar illnesses originating from consumption of common contaminated food or water (Bennett et al., 2013). Surveillance of foodborne diseases plays vital role in the timely detection and reporting of foodborne disease outbreaks and their control. Early identification and eliminating

bacteria from source of the outbreak is becoming ever more important as countries move towards industrialization (Käferstein et al., 1997), increasing production from home to industry. There are many synthetic, chemical and natural compounds which could stop the growth of pathogenic microorganism in foods, thereby preventing outbreak of food borne disease. Additionally, these compounds increase in the shelf life and value added to food products. Bacteriocin is a natural compound(s) that has been highly studied due to its value added properties and recognized as safe for consumption.

2.4 Bacteriocin

Bacteriocin are ribosomaly synthesized, short peptides or antimicrobial peptides (AMP) with bactericidal or bacteriostatic activity on closely related species. Bacteriocin differ with antibiotics in several aspects, as mention by (Cleveland et al., 2001). Though bacteriocin are produced by bacteria and possess antimicrobial properties, but bacteriocin are distinguished from antibiotics to avoid misunderstanding and concern with therapeutic/clinical antibiotics that can potentially cause allergic reactions in humans (Cleveland et al., 2001). Bacteriocin may be either chromosomally or plasmid encoded, for instance Plantaricin 423 is plasmid encoded, while Plantaricin ST31 is chromosomally determined (Todorov, 2009).

2.4.1 General classification of bacteriocin

Bacteriocin are classified into four classes on the basis of their mode of action, size, structure and function by Klaenhammer (1988);

i. Class I (Lantibiotics)-These group of bacteriocin are composed of small one or two active peptides of approximately 5 kDa Rea et al. (2011). Class I consists of small, post-translationally modified peptides which are characterized by the presence of modified thioether amino acids such as lanthionine, methyllanthionine and α, un-saturated amino acids such as dehydroalanine and dehydrobutyrine and are usually referred to as lantibiotics. Type A lantibiotics (21 to 38 amino acids), usually act by depolarizing cytoplasmic membrane. A common example of this group is nisin, produced by *Lactococcus lactis* subsp. *Lactis* (Chen and Hoover, 2003). The type B lantibiotics are slightly smaller

with >19amino acids and act by enzymatic action and interferes cell wall biosynthesis (Klaenhammer, 1988)

ii. Class II (Non-lantibiotics)-These group f bacteriocin are generally unmodified peptides of <10 kDa. Class II comprise a very large group of heat-stable unmodified peptide bacteriocin which can also be further subdivided. It has been shown that several LAB and *Bacillus* produce multiple bacteriocin (2–3 bacteriocin) while most bacteriocin producers synthesize only one bacteriocin (Ennahar et al., 2000).

iii. Class III-These bacteriocin are generally >30 kDa and the least studied group. It consists of heat-labile proteins such as helveticin J and enterolysin A.

iv. Class IV- Usually regarded as contaminants with large complexes of chemical moieties, such as, carbohydrates or lipids required for activity.

Bacteriocin, the anti-microbial substances of LAB and *Bacillus* have been widely studied in recent years because it is relatively safe with GRAS property and has been proposed as food bio-preservative. Moreover, there is increase in negative perception towards chemical preservative and consumers favor foods with few or no chemical preservatives. Therefore, either bacteriocin producing starter/protective cultures or their bacteriocin have received much interest as natural food bio-preservative. The following prerequisites should be achieved by any natural preservative to be used commercially (Gautam and Sharma, 2009, Jeevaratnam et al., 2005); the bio-preservative should be non-toxic, it should be allowed to be used by food safety authorities. Also, the stability to processing and storage should be considered. It does not affect the aroma or taste of foods and have efficacy at low concentrations.

2.4.2 Mode of action of bacteriocin

The mechanism of activity of bacteriocin or BLIS producing strains are mostly limited to Gram positive bacteria and only few bacteriocin has effect on Gram negative bacteria. Bacteriocin belonging to class I are mostly bactericidal to sensitive cells, except for few bacteriocin being bacteriostatic. The smallest bacteriocin produced are

approximately 3 kDa. The mechanism of action of antimicrobial peptides or bacteriocin seems to involve multiple targets. In Gram positive bacteria nisin has been shown to act on energized membrane vesicles to disrupt the proton motive force (PMF), inhibit uptake of amino acids, and causes release of accumulated amino acids (Jung and Sahl, 1991). The dissipation of PMF was identified as the most common for mode of action for bactericidal activity of many bacteriocin. Bacteriocin may dissipate, either or both trans-membrane potential and/or pH gradient, the components of PMF. The antimicrobial peptides or bacteriocin are active against Gram positive bacteria compared to the Gram negative, due to the difference in composition of outer cell membrane. Gram negative bacteria possess an outer membrane composed primarily of lipopolysaccharides (LPS), which act like semipermeable layer by preventing the free diffusion of large molecules above 0.6 kDa while the smallest known bacteriocin is of 3 kDa size (Klaenhammer, 1993, Hastings et al., 1991). However, some bacteriocin possess ability to be transported through receptors present on outer membrane as well as on inner membrane of Gram negative bacteria (Cotter et al., 2013) subsequently bringing to cell death. Although Gram negative bacteria are not susceptible to bacteriocin, but removal of LPS make them sensitive towards bacteriocin (Stevens et al., 1991).

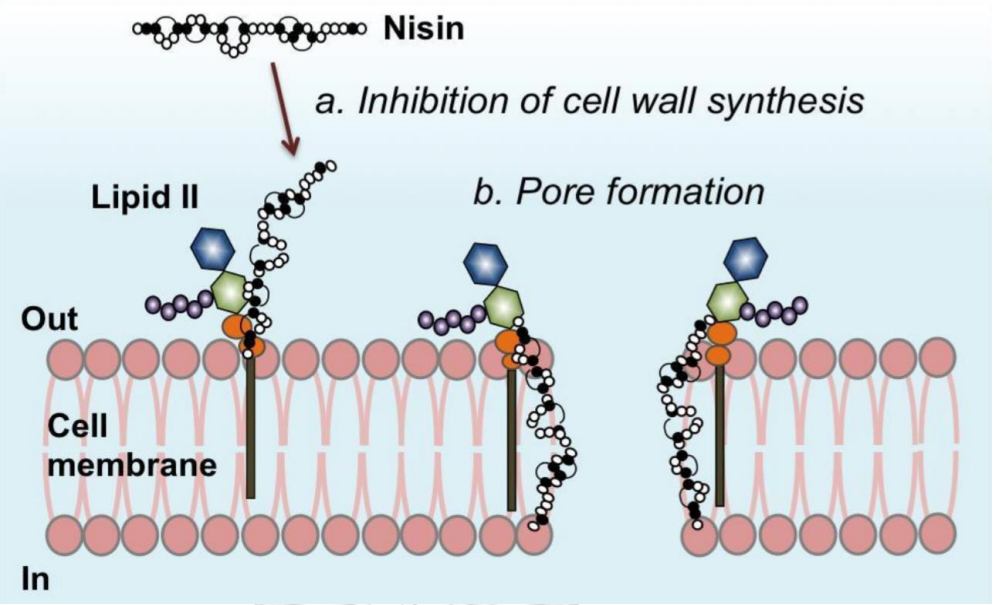


Figure 2.2 Dual mode of action of class I bacteriocin

Class I bacteriocin, commonly studied nisin, have a dual inhibitory mechanism in killing their target cells through (a) firstly inhibition of direct cell wall synthesis by blocking the essential building materials and (b) pore formation in cell membrane leading to efflux and dissipation of PMF (Breukink et al., 1999, Wiedemann et al., 2001, Perez et al., 2015).

Apart from disruption of PMF, bacteriocin may act by inhibiting uptake of amino acids and activate to release out from the cell (Hugenholtz and De Veer, 1991), or it may lead to exclusion of potassium ions and depolarization of the cytoplasmic membrane (Abee et al., 1995). Therefore, generally bacteriocin are found to be active against many important human pathogens particularly Gram positive bacteria. Though LAB is considered a major bacteriocin producer, the members of *Bacillus* group are also known to be good producers of antimicrobial substances such as peptide and lipopeptide antibiotics, as well as numerous bacteriocin (Chalasani et al., 2015).

2.5 *Bacillus*

Bacillus species are Gram positive and endospore-forming bacteria. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants (Baron, 1996). *Bacillus* includes a vast species including harmless saprophytes and few pathogenic strains. *Bacillus anthracis*, the pathogenic agent of anthrax disease, is the most important pathogen of the group followed by *B. cereus* that cause food poisoning (Thwaite and Atkins, 2012). The other *Bacillus* strains commonly isolated are *B. subtilis, B. licheniformis B. amyloliquefaciens, B. pumilus, B. atrophaeus, B. altitudines and B. stearothermophilus.* Amongst them, *B. subtilis* is considered industrially important spp., also used in the production of *Natto*, a traditional Japanese dish of fermented soya beans (Kunst et al., 1997) and also known to produce variety of bio-active peptides (Abriouel et al., 2010).

2.5.1 Bacteriocin from *Bacillus* spp.

Bacillus represents an attractive representative genus for the identification of bacteriocin because it has a long history of safe use (GRAS) (de Boer Sietske and Diderichsen, 1991, Sanjukta et al., 2015, Kaewklom et al., 2013). It is also considered to be the second most important bacteriocin producer following LAB (Abriouel et al., 2010) and bacteriocin from *Bacillus* display wider spectrum of inhibitory activities, extending to few Gram negative bacteria (Xie et al., 2009). The other advantage is *Bacillus* has its ability for formation of dormant spore under limited or unavailable nutrients and in harsh environments. The *Bacillus* spore may be metabolically inactive, but during due process of spore formation it produce enzymes that have antibacterial effect and has incident for re-reproduction during favorable condition to produce bacteriocin (Phillips and Strauch, 2002).

Bacteriocin from *Bacillus* species, together with those from LAB, are gaining considerable attention for applications in human and animal health. Lim et al. (2016) isolated the RX7 strain, in local soil sample and known to be *B. amyloliquefaciens* identified by biochemical profiling and 16S rDNA sequencing, which is active against the food pathogen *L. monocytogenes*. The activity of the antimicrobial substance from

B. amyloliquefaciens RX7 for proteases was indicative of its proteinaceous nature, leading us to classify it as a bacteriocin. This compound exhibited broad spectrum antibacterial activity to both Gram positive and Gram negative bacteria. Other well-characterized bacteriocins from *B. amyloliquefaciens* are amylolysin and amylocyclicin. Guo et al. (2016) tested food samples, such as different kinds of cheese and vegetables from stores (Columbus, OH) and were studied for isolates with production of antimicrobial property. They isolated new bacterial strain, OSY-7LA, and identified as *Bacillus atrophaeus* by 16s RNA sequencing. The strain produced secondary metabolite, bacteriocin like inhibitory substance which is active against *Listeria* strains and antibiotic resistant MRSA strains.

The two most popularly produced bacteriocin by *B. subtilis* and other closely related species are subtilosin and subtilin. Subtilosin is produced during vegetative growth and production is stopped during sporulation (Babasaki et al., 1985). Subtilosin is circular antimicrobial peptide (Velho et al., 2013), ribosomaly synthesized 35 amino acids cyclic peptide, known for its active antimicrobial action (Marx et al., 2001). Subtilin is also lanthionine containing active peptide antibiotic, class I type of bacteriocin. Subtilin is active to a wide range of bacteria, either by inhibiting the cell wall synthesis or the formation of membrane pores. These formation of pores on cell membrane leads to depolarization of the membrane and thus, to swift cell death (Banerjee and Hansen, 1988).

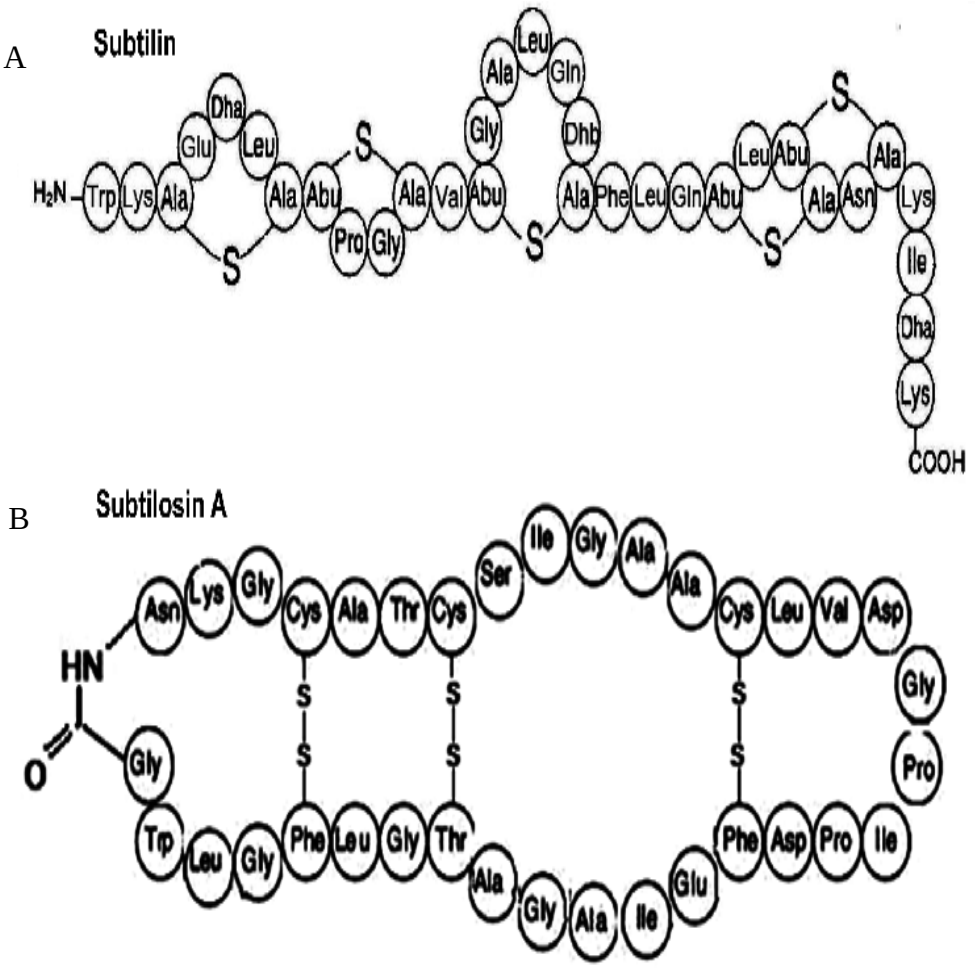


Figure 2.3 Subtilin and Subtilosin

Fig A. Subtilin structure (molecular formula $C_{148}H_{227}N_{39}O_{38}S_5$, MW 3320.979 g/mol). Subtilin is a lanthionine containing peptide (32 amino acids) bacteriocin.

Fig B. Subtilosin (molecular formula $C_{129}H_{208}N_{36}O_{41}S_3$, MW 3015.474 g/mol) (Kawulka et al., 2004, Stein, 2005). Subtilosin is 35 amino acids, cyclic peptide (Marx et al., 2001).

2.5.2 Genetic Expression

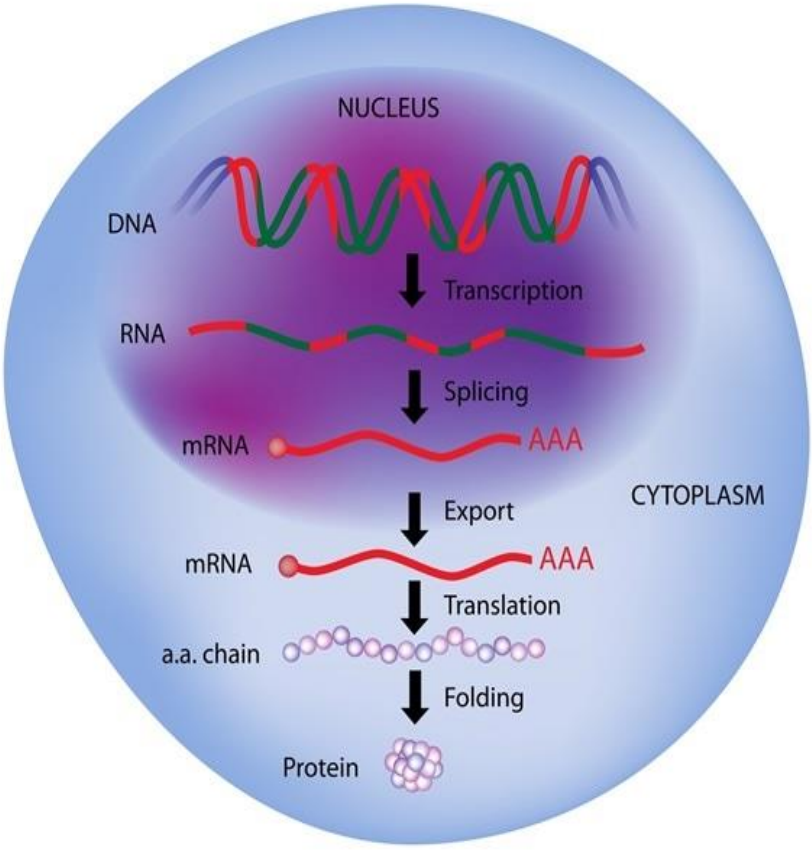


Figure 2.4 Gene Expression steps

Gene expression is a sequence of natural approach that converts the information encoded in a gene to active and functional product such as a functional protein. Firstly, DNA molecules are transcribed into their corresponding RNA copy. This process is called transcription and the enzyme involved is called DNA-dependent RNA polymerase. The RNAs (mRNA, tRNA) translated into a protein in presence of enzyme aminoacyl tRNA synthetase through the process of translation. Finally, numerous amino acid chain is conferred together to form functional protein as an end product of gene expression process.

The genes encoding bacteriocins are usually known to be located in plasmids and transposons, occasionally it is also found in genomic islands. Studies also found that,

the other releated function involving in cell self protection, such as immunity protein (LanFEG) (Lundström, 1912, Stein, 2005), regulatiory protein, and transporter gene for bacteriocin (Nes et al., 2007) are also expressed along during bacteriocin production. The expression of common *Bacillus* bacteriocin; subtilin biosynthesis is progressed through positive feedback mechanism and know to have a dual expression mechanism (Stein et al., 2002). The other important genes involved are subtilin structural gene *spaS* (Banerjee and Hansen, 1988) and subtilosin A structural gene *sboA* (Zheng et al., 2000), biosynthesis and transport genes *spaBTC* (Klein et al., 1993), and histidine kinase SpaK represents regulatory gene.

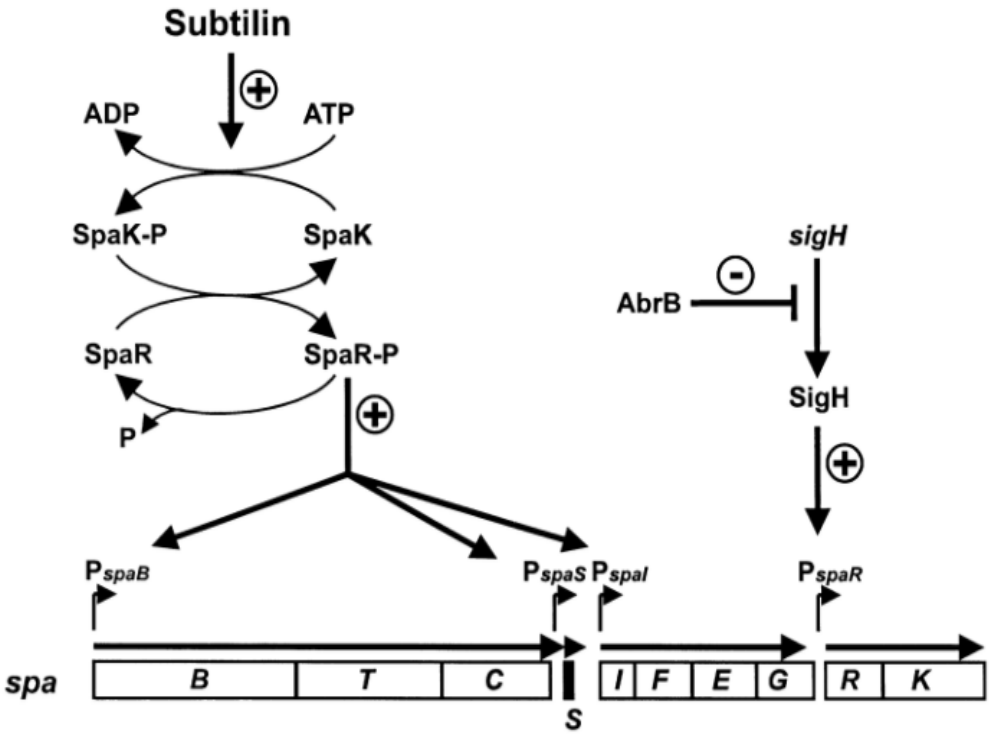


Figure 2.5 Dual control of subtilin biosynthesis and immunity

Source: Stein et al. (2002)

Stein et al. (2002) studied the bacteriocin biosynthesis and immunity in *B. subtilis*, they found expression of *SpaB* with high intensity band when conducted by Northern hybridization from mid-logarithmic growth phase. The researchers concluded

that the signal for gene expression disappeared after 5-6 hours. Thus, the samples taken over 6 hours could show falsely negative results, unless it is over-expressed in the system.

Finally the activity of bacteriocin gene expression can be estimated for antimicrobial activity by measuring the inhibitory zone during stab culture method due to the activity of the functional proteins (Moraes et al., 2010).

2.5.3 Bacteriocin inhibitory action

The deferred antagonism or spot-on-the-lawn culture method is commonly used technique to detect the inhibitory action due to the production of antimicrobial peptides produced by bacteria (Moraes et al., 2010, Marshall et al., 2010). The targeted strain of bacteria is seeded on selected agar plate and then stabbed or over layered with the bacteriocin producer strain. The zone of inhibition is examined after 24 hours after incubation at 37°C. Both, the cell co-culture and cell free supernatant (Shin et al., 2008) can be used to study the inhibitory action. After antagonist activity is been detected, supplementary tests; gene encoding bacteriocin production, gene expression are conducted. Moreover, pathogenicity testing is required to be conducted prior to application into food system for the isolates with inhibitory activity on pathogenic bacteria and showing gene expression.

2.6 Safety evaluation

Microbial peptides and microbial culture used in food processing or preparation not only have desired activity but also other metabolites from the production strains that is not limited to desired activity itself (Pariza and Johnson, 2001). The safety of microbes, such as pathogenicity and toxigenic potential is primary consideration to be evaluated before considering as food grade. The Joint Food and Agriculture Organization (FAO) of the United Nations/World Health Organization (WHO) Expert Consultation (Venugopalan et al., 2010) stated the minimum safety assessment of the food microorganism should be determined for any toxin production and hemolytic potential prior to addition as preservatives, starter or protective culture. The European Food Safety Authority (EFSA) stated about the qualified presumption of safety (QPS)

concept about microorganism in food and feeds (Leuschner et al., 2010). It allows the microorganism to be food grade after accessing for safety evaluation, body of use (history of use, clinical aspects, literature review, possible safety concerns (pathogenicity) as mentioned in EFSA (Anadón et al., 2006).

2.6.1 Allergens

Biogenic amines (BA) are naturally occurring low molecular weight compounds associated and carrying out various biological activities. However, biogenic amines are also known to be allergens and can cause hypersensitivity reaction related health problems to sensitive human (Lonvaud-Funel, 2001). Histamine forms are one of the major biogenic amines in a variety of foods, including raw fish, wine, cheese, fermented meat and fish products (Santos, 1996).

The common biogenic amine histamine (precursor histidine) produce physiological effects; act as neurotransmitter, secret local hormone, regulation of immune response, allergic reactions and toxicological effects causing headaches, sweating and other symptoms (Ladero et al., 2010). The other commmon biogenic amine is tyramine (precursor tyrosine) causing physiological and toxicological effects as such nausea, vomiting, increasing heart rate, increasing sugar and also hypertension (Ladero et al., 2010, Spano et al., 2010). Putrescine and Cadaverine (precursor Ornithine and Lysine) are occasionally produced causing, mild allergic reactions and increasing heart rate (Spano et al., 2010).

Moreover, in salty fermented fish, several report demonstrated that during the decomposition of such salty fermentation, histamine formed are in significant amounts due to bacterial decarboxylation of available histidine, produced by decarboxylation of histidine by the enzyme histidine decarboxylase (*hdc*) (HDC; EC 4.1.1.22). Subsequently, tyramine via tyrosine decarboxylase (*tyrdc*) (TDC; 4.1.1.25) and putrescine via ornithine decarboclyase *(odc*) (ODC; 4.1.1.17). There are various methods which are principally based on differential media signaling pH upon production of BA. Enzymatic method dependent upon production of hydrogen peroxide, chromatography (TLC, HPLC) are used for qualitative and quantitative study (Marcobal et al., 2006).

Early and timely detection of possibly BA producing bacteria in food industry is critical, due to its possibilities of causing food poisoning (Landete et al., 2007). Therefore, a molecular method with higher sensitivity has been developed for detection of these BA, which detects the presence of the gene encoding production of biogenic amine enzyme. Moreover, it has advantage over cultural method due to its simplicity to use, early and rapid detection, thereby preventing accumulation of BA. Molecular methods are becoming an alternative to traditional cultural method and replacing it. This detect histamine producing gene, several oligonucleotide primers (CL1, CL2, JV16HC, and JV17HC) were designed and widely used (Jeune et al., 1995). A multiplex PCR assay for detection of tyramine, histamine and putrescine was later developed (Coton et al., 2004, Marcobal et al., 2012).

2.6.2 Cytotoxic effect

Cell cytotoxicity and acute toxicity refers to the potential of certain chemicals or mediator cells to cease living cells and often calculated as IC_{50} (maximal inhibitory concentration) (Meena, 2009). Cytotoxic effects of *Bacillus* spp. on human colorectal adenocarcinoma cell line or also called as Caco2 cells and Vero cell line are used to screening pathogenicity. Caco2 cell line is an intestinal model where it has direct contact with food (Er et al., 2015). Vero cell is a tissue culture cell line derived from African green monkey kidney epithelial cells (Casem, 2016) used to study the cytotoxic effects.

It is known that the many species of *Bacillus* including *B. cereus,* causative agent for food poisoning produce certain types of toxins. The genes that function for the successful colonization and survival of the bacterium in or cause damage to the host cells are considered as virulence or pathogenicity gene (Thomas and Wigneshweraraj, 2014). Several virulence genes are located in bacterial plasmids and produce toxins. Production of stable toxins, such as surfactin produced from *B. subtilis* (Taylor et al., 2005), amylosin produced by *B. amyloliquefaciens* (Mikkola et al., 2007), pumilacidin from *B. pumilus* and lichenysin from *B. licheniformis* (Nieminen et al., 2007) were proposed to be the origin of the cytotoxic factors in some strains of *Bacillus* (Panel, 2011).

2.6.3 Hemolysin

Members of the genus *Bacillus*, such as *B. cereus, B. thuringiensis* and *B. mycoides* are known to produce certain hemolysins that contributes to the pathogenicity of these species, otherwise known to be saprophytic soil-borne bacteria (Mukry et al., 2010). Different types of hemolysins produced by *Bacillus* group (Cowell et al., 1976, Budarina et al., 1994, Lund et al., 2000) includes:

i. Cereolysin (hemolysin I)
ii. Cereolysin AB
iii. Hemolysin II
iv. Hemolysin III
v. Hemolysin BL (HBL)
vi. Cytotoxin K

Conventional culture based laboratory method are used for screening of hemolytic activity, the overnight cultures from mid stationary phase spotted and incubated on plates containing nutrient agar supplemented with red blood cells 5-6% horse/human/rabbit or sheep blood, at pH 7.0. Clear zone of inhibition or also known as hemolytic zone around each spot was noted after 24 hours of incubation at 37°C. The different pattern of hemolysis produced could be either alpha (α), beta (β) or gamma (λ) depending on zone of inhibition.

2.7 Molecular technique for bacterial identification

2.7.1 Sequencing analysis and Phylogenetic

The interdisciplinary fields, involving ideas from physics, mathematics, biology, and computer science, applied to compute for analysis to the capture and interpretation of biological data is called as Bioinformatics (Bayat, 2002). Basic Local Alignment Search Tool (BLAST) is bioinformatics tool of NCBI widely used method for sequence similarity search from gene bank. The BLAST program can be used for either protein or nucleotide sequence analysis (Koonin and Galperin, 2003). Basically DNA are transcribed to RNA, and RNA translated to functional protein (nucleotides). The nucleotide is the basic building material of nucleic acid. The sequencing results is achieved by comparison of these nucleotide pairs. It is achieved by comparing the reference database sequences through high scoring segment pair (HSPs) methods, which is relatively faster and finding the corresponding homology pair or identification (Krauthammer et al., 2000) from the gene bank.

Phylogenetic is the scientific study of phylogeny. Phylogeny pertains to the evolutionary history of a taxonomic group of organisms, over the period of time, addressing the query between species, drawing relationships among species or genes, demographic changes and migration patterns of species and also helping address certain biological inquiries (Yang and Rannala, 2012). A phylogeny is a tree that connects via nodes and branches. Branches represents the persistence of a genetic heredity through time, and each node represents the birth of a new heredity (Yang and Rannala, 2012). The neighbor-joining (NJ) (Saitou and Nei, 1987) and unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) are the popular tree-building methods for distance matrices.

2.7.2 Denaturing Gradient Gel Electrophoresis

Reverse Transcriptase Polymerase chain reaction-denaturing gradient gel electrophoresis (Rev-T-PCR-DGGE) is performed by amplification of ribosomal RNA and this amplicon run on a polyacrylamide gel containing an increasing gradient of denaturants (Chen et al., 2008), as introduced by Muyzer et al. (1993). DGGE is a method used for segregating different length of DNA/RNA fragments according to their mobility under increasingly denaturing conditions (usually increasing formamide/ urea concentrations). The other process involved are PCR of the fragment(s) for analysis, gel preparation, running gel at low voltage, staining and viewing. According to Sheffield et al. (1989) DGGE method analysis can detect up-to 50% of the sequence variants from DNA fragments up to 500 bp and this can be further increased to almost 100% by the attachment of a GC rich sequence, so called GC clamp, to one side of the DNA fragment. Fontana et al. (2005) from Argentina, in their research about microbial diversity in dry fermented sausage following PCR-DGGE technique, using three sets of primer Vlf-Vl, Bact-0124-Uni-0515r and V3f-Uni-0515r, targeting the V1, V1-V2 and V3 region of 16srRNA gene, found the best result from amplifying V3 region of 16srRNA using primer V3f(GC)-Uni-0515r. Therefore, V3 region of 16srRNA, 338F/517R primer pairs with GC clamp was used in the parenthesis.

Kary Mullis in 1983 (Bartlett and Stirling, 2003) developed polymerase chain reaction (PCR) as a molecular biology technique used to amplify the single segment/fragment of DNA into millions of copies for detection (Mullis et al., 1986). Nested PCR assays is performed using two pairs of universal primers. This technique has increased detection sensitivity over 100 folds (Gundersen and Lee, 1996). The first set of PCR amplification is group specific primers. The second PCR round was performed with a second set of universal bacterial primers and served to reduce and equalize the length of the specific fragments or region and to add a GC-clamp, necessary for performing bacterial ecology study by DGGE analysis (Boon et al., 2002).

CHAPTER 3

METHODOLOGY

The equipments/instruments, materials, chemicals, standard bacterial strains, PCR reagents, and primers used and samples analyzed for this research purpose are listed as below:

3.1 Materials and Instruments

3.1.1 Cultural Medias

i. De Man, Rogosa and Sharpe (MRS) agar (Himedia, India)
ii. Skim milk powder (Himedia, India)
iii. Nutrient agar (Himedia, India)
iv. Nutrient broth (Himedia, India)
v. Potato Dextrose Broth (PDA) (Himedia, India)
vi. Peptone (Merck, Germany)

3.1.2 Instruments

i. Autoclave (Tommy SX-700, Meditop, Thailand)
ii. Biosafety cabinet (Class II cabinet, Telstar, Thailand)
iii. Bio spectrometer (eppendrop®, Germany)
iv. Centrifuge (Hermle Z36HK, HERMLE Labortechnik GmbH, Germany)
v. Centrifuge (Mikro 22R, Germany)
vi. Colony Counter (Gallenkamp, England)
vii. Deep freezer (Sanyo Biomedical freezer, Japan-Thailand)
viii. DGGE chamber (BioRad™ Universal Mutation detector, Singapore)
ix. Electronic balance (Mettler Toledo, Swizerland)
x. Electrophoresis gel chamber (HU413L, United Kingdom)
xi. Electrophoresis power supply (Amersham pharmacia, Bitech, Sweden)
xii. Gel documentation chamber (SYNGENE Bio-imaging, USA)
xiii. Hot air Oven (Heraeus, Germany)
xiv. Hot air Oven (Memmret, Germany)
xv. Micropipette (Pipet-Lite XLS, Rainin®, Mettler Toledo, Thailand)
xvi. Micro plate Reader (ASVS, Biochrom, England)
xvii. pH meter (CyberScan® pH 1000 meter, Eutech instruments, Netherland)
xviii. Stomacher (AES Labotorie, France)
xix. Shaker (Innova™ 2000, New Brunswick Scientific, Thailand)
xx. Refrigerator (Mitsubishi, Thailand)
xxi. Thermal Cycler (BioRad T100™ Singapore)

xxii. UV transluminator (Vilber Lourmat, France)
xxiii. Vortex (Vortex Genie 2, Scientific Industries, Thailand)
xxiv. Water bath (One 7, Memmert, Germany)
xxv. Whatman® no.1 filter paper
xxvi. Water bath (Scientific Promotion, Thailand)
xxvii. 100-1000µl Blue Pipette (Thermo Fisher Scientific, Mexico)

3.1.3 Chemicals and Reagents

i. Agarose basic (Applichem, Spain)
ii. Ammonium Persulphate (Vivantis, Malaysia)
iii. Bis/Acryl™ (Life Science, USA)
iv. Formamide (Merck, Germany)
v. Gelatin powder (Ajax Finechem, Zew Zealand)
vi. Methanol (Fisher Scientific, England)
vii. Potassium Ferrocyanide (LOBA Chemie, India)
viii. Sodium Chloride (QReC®, Zew Zealand)
ix. Sodium Dodecyl Sulphate (Vivantis, Malaysia)
x. Silver Nitrate (SCRC, Zew Zealand)
xi. Tris (Vivantis, Malaysia)
xii. Temed (BioRad, USA)
xiii. Urea (BioRad, USA)
xiv. Zinc Acetate (Ajax Finechem, Zew Zealand)

3.1.4 PCR Reagents

i. DNA Tag polymerase (Vivantis, Malaysia)
ii. dNTPs (Vivantis, Malaysia)
iii. Ethidium Bromide (applichem, Spain)
iv. Ladder (Vivantis, Malaysia)
v. Magnesium Chloride (Vivantis, Malaysia)
vi. Total RNA extraction kit (Vivantis, Malaysia)
vii. Buffer (Vivantis, Malaysia)

3.1.5 Primers

Table 3.1 List of primers

Primer	Primer sequence	Reference
Bacterial V3 regions of 16SrDNA	338FACT CCTACG GGA GGC AGC AG	Wei et al. (2013)
	518RATTACC GCG GCT GCT GG	
GC Clamp	5′-GCC GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG-3′	Wei et al. (2013)
Subtilin (*Spa*)	FCAAAGTTCGATGATTTCGATTTGGAT GT	Klein et al. (1992)
	RGCAGTTACAAGTTAGTGTTTGAAGG AA	
Subtilosin (*Sbo*)	FCGCGCAAGTAGTCGATTTCTAACA	Stein et al. (2004)
	RCGCGCAAGTAGTCGATTTCTAAC	
Housekeeping gene BA-rpoB	F5'-GAC GAT CAT YTW GGA AAC CG-3'	Ko et al. (2004)
	R5'-GGN GTY TCR ATY GGA CAC AT-3'	
Biogenic amine Histamine	JV16HC, 367bp AgA Tgg TAT TgT TTC TTA Tg	Le Jeune et al. (1995)
	JV17HC AgA CCA TAC ACC ATA ACC TT	
	HDC3, 435 bp gAT ggT ATT gTT TCK TAT gA	Cotton et al. (2010)
	HDC4 CCA AAC ACC AgC ATC TTC	
Tyramine	TD2, 1100 bp ACA Tag TCA ACC ATR TTg AA	
	TD5 CAA ATg gAA gAA gAA gTA gg	
	TDC1, 720 AAC TAT CgT Atg gAT ATC AAC g	Fernandez et al. (2007)
	TDC2 Tag TCA ACC ATA TTg AAA TCT gg	
	TDC-F, 825 bp Tgg YTN gTN CCN CAR ACN AAR CAY TA	De las Rivas et al. (2005)
	TDC-R ACR TAR TCN ACC ATR TTR AAR TCN gg	
Putrescine	PUT1-F, 1440 bp TWY MAY gCN gAY AAR CAN TAY YYT gT	
	PUT1-R ACR CAN AGN ACN CCN gNg gRT ANg g	
	PUT2-F, 624 bp ATH WgN TWY ggN AAY ACN ATH AAR AA	
	PUT2-R gCN ARN CCN CCR AAY TTN CCD ART C	

3.1.6 Bacterial culture

i. *Staphylococcus aureus* ATCC 25923 (Faculty of Science, Chulalongkorn University)
ii. *Staphylococcus aureus* DMSc 6538 (Department of Medical Science, Ministry of Public Health, Thailand)
iii. Isolate from food *Staphylococcus aureus* FT30-7 isolate (Faculty of Science, Chulalongkorn University)

3.2 Samples

Plara, soya sauce, cheese and bamboo shoot pickle were the food samples collected for this study purpose. Their source and collection period are as mentioned.

Table 3.2 Soya Sauce samples

Sl. No	Code	Sampling station
1	SS1	Final product from Nakorn patom, Thailand
2	SS2	Final product from Nakorn patom, Thailand
3	SS3	4-month product from Nakorn patom, Thailand
4	SS4	4-month product from Chacheongsao, Thailand

Table 3.3 Plara samples

Sl. No	code	Sampling station
1	P1	Final product from Nongkhai, Thailand
2	P2	Final product from Nongkhai, Thailand
3	P3	1-month product from Nakhon Rachasima, Thailand
4	P4	Final product from Nakhon Rachasima, Thailand
5	P5	Final product from Nakhon Rachasima, Thailand
6	P6	Final product from Ubon Ratchathani, Thailand
7	P7	4-month product from Chaiyaphum, Thailand
8	P8	Final product from Sakon Nakhon, Thailand
9	P9	Final product from Vientiane, Laos
10	P10	Final product from Vientiane, Laos
11	P11	Final product from Udon Thani, Thailand
12	P12	Final Product from Udon Thani, Thailand
13	P13	Final product from Udon Thani, Thailand
14	P14	Final product from Udon Thani, Thailand
15	P15	Final product from Nongkhai, Thailand
16	P16	Final product from Ubon Ractchathani, Thailand
17	P 17	Final product from Nongkhai, Thailand

Table 3.4 Food sample for testing

Sl. No	Sampling station
1	Bamboo shoot pickle from Tsirang, Bhutan
2	Cheese from Local farm from Bumthang, Bhutan

3.3 Methodologies

3.3.1 Sample collection and preparation

Salty fermented foods, Plara was collected from several manufacturers and production steps from Thailand and Laos, as listed in the table 3.3. Soya sauce samples were collected from both the industrial processing unit and the traditional manufacturer from Thailand, as listed in the table 3.2. All samples were collected in a sterile closed container and transported to the laboratory as soon as possible.

3.3.2 Investigation and isolation of Halophilic bacteria in salty fermented foods

3.3.2.1 Molecular method

RNA Extraction: To study the bacterial communities in soya sauce, and Plara samples and their role in the fermentation system, the bacterial RNA were directly extracted from food samples and purified using commercial kits (GT-1, Total RNA extraction, Vivantis, Malaysia) (Appendix A1). The purified RNA were converted to complementary DNA (cDNA) by reverse transcriptase reaction (Total RNA extraction kit, Vivantis, Malaysia). The first set of reverse transcription reaction was performed using thermal cycler (BioRad T100^{TM} Singapore) and standard PCR tubes, containing a total of 10µl mixture with dNTPs (10U), Oligo d (40um), hexamer (50ng) and 2µL (10-50ng/µL) programed initially at 65°C, 5 min. Then, followed by 42°C, 60 min with addition of 10X buffer, reverse transcriptase (200U) and RNA free water making the total volume up to 20µl.

Polymerase Chain Reaction (PCR): Nested PCR was performed on cDNA templates. The first set of universal primer used is 27F (AgT TTg ATC CTg gCT Cag) and 1492R (ggC TAC CTT gTT Acg ACT T) (Frank et al., 2008). Secondly, for DGGE analysis of true bacterial community study, universal bacterial PCR primer set targeting the 16s rRNA V3 region (338F: ACT CCTACG GGA GGC AGC AG and 518R: ATTACC GCG GCT GCT GG) was used (Wei et al. (2013). The GC clamp attached at 518R (5′-GCC GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG-3′) Wei et al. (2013). Amplification was done in a standard reaction mixture of 50 µL, 4 µL DNA (10-50ng/µl), 0.1 mM of each primer, 1.5mM $MgCl_2$, 0.1mM dNTPs mix and 2 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to an initial cycle of denaturation (94 °C for 2 min), followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 30 s), ending with extension at 72°C for 7 min, in a DNA thermal cycler (BioRad T100^{TM} Singapore).

DGGE analysis and sequencing: DGGE analysis was performed following electrophoresis technique in DGGE chamber, at 120 V for 4 hrs, in running buffer (0.5× Tris-acetate-EDTA) maintained at constant temperature of 60 °C using a 16 cm×16 cm×1 mm 8 % (w/v) polyacrylamide gel acrylamide-bisacrylamide (37.5:1) (Life Science, USA) with a 20 % to 40 % denaturing gradient of urea plus formamide using DCode universal mutation detection system (Bio-Rad). Gels were stained with ethidium bromide (Applichem, Spain) and photographed under UV transluminator (Vilber Lourmat, France) (Heilig et al., 2002). DGGE bands on gel were extracted, transferred to a 1.5mL micro centrifuge tube with 100 µL sterile water for elusion and kept overnight at 4°C. Finally, 2µL aliquot of supernatant containing DNA was re-amplified and sequenced by commercial manufacturer (Macrogen, Korea). Sequenced results were analyzed by performing a nucleotide BLAST search of Gene Bank (NCBI).

3.3.2.2 Culture method

Nutrient agar (Himedia, India) supplemented with 5% NaCl (QReC®, New Zealand) were selected for cultural isolation of bacteria from Plara and soya sauce samples. Nutrient agar was prepared as per the manufacturer instruction (Appendix B1). 10 grams of samples was weighed and mixed to the diluent 90mL of 0.1% peptone water (Merck, Germany) and then serially diluted. 0.1mL sample was poured on the

plate and incubated at 37ºC for 24hrs. The colony numbers were counted and the bacterial colonies for further investigation were selected following Harisson dish techniques (Harrigan, 1998). The bacterial colonies were stored using 20%V/V glycerol stock at -21°C.

3.3.2.3 Salt tolerance test

The bacterial colonies were screened for their salt tolerance property using nutrient broth containing 1, 5, 10, 15 and 20 % NaCl. The cells were cultured in 96 well micro-plate sample tray and the culture turbidity were then measured at 600nm, after incubation at 37°C for 24 hours (Tanasupawat et al., 2002).

3.3.3 Investigation of inhibitory effect on *S. aureus* and gene expression

3.3.3.1 Screening of potential Bacteriocin producer

The halophilic bacteria was investigated for inhibitory property on *S. aureus* using spot-on-lawn deferred antagonism technique (Shin et al., 2008, Moraes et al., 2010). The isolates culture of mid stationary phase was spotted on nutrient agar supplemented with 5% NaCl and seeded with *S. aureus* culture. Two pathogenic (ATCC 25922 and DMSc 6358) and one food isolate (FT 30-7) strain of *S. aureus* were used for this process. A mix/cocktail *S. aureus* was prepared by adding equal amount 1:1:1 volume (0.1 OD at 600 nm) of three different *S. aureus* strains grown in 5% NaCl nutrient broth overnight respectively, modified from Honório et al. (2015). The plates were incubated at 37°C for 24 hours. After incubation, the isolates showing clear/inhibition zone (diameter in mm) on the lawn were selected for further investigation of inhibitory function. The test was repeated in triplicate and the means were analyzed using SPSS at $p < 0.05$, compared using Duncan test.

3.3.3.2 Nucleotide sequencing analysis

The isolates demonstrating inhibitory effects were considered as a potential bacteriocin or bacteriocin like substance producer. These isolates were identified for DNA sequencing analysis. The isolates were cultured on NA plate supplemented with 5% NaCl and grown at 37°C for 24 hours. Bacterial DNA was extracted following crude cell DNA extraction process (Dashti et al., 2009) (Appendix A3).

The nucleic acid sequences were chosen from the conserved regions of 16S rDNA. PCR was performed using primer set 338F/519R (Mao et al., 2012), in 50-µL reaction mixtures using 2 µL DNA (10-50ng/µL), 0.1 mM of each primer, 1.5mM $MgCl_2$, 0.1mM dNTPs mix and 2 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to an initial cycle of denaturation (94 °C for 2 min), followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 30 s), ending with extension at 72°C for 7 min, in a DNA thermal cycler (BioRad T100^{TM} Singapore). 10µL of PCR products were directly applied onto 1.5% (w/v) agarose gel in 1% TAE buffer containing 2M Tris base, 1M glacial acetic acid, 0.5 M EDTA, pH 8.0 to 1000mL distilled water. Electrophoresis was performed at a constant voltage of 200V for 30 minutes (Electrophoresis gel chamber; HU413L, United Kingdom and Electrophoresis power supply, Amersham pharmacia, Bitech, Sweden). After electrophoresis, gel was stained with 1% ethidium bromide (Applichem, Spain) and photographed under UV transluminatior (Vilber Lourmat, France). Thus, this 16S rDNA region amplified PCR product was send to commercial manufacturer for sequencing analysis (Macrogen, Korea) after cleaning. The sequencing data was analyzed with nucleotide BLAST program of NCBI. Finally, phylogenetic tree was constructed using Geneious programme.

3.3.3.3 Gene encoding bacteriocin analysis

The isolates with inhibitory action on *Staphylococcus* tested strains were further subjected to evaluate for gene encoding bacteriocin production, after the bacteria were identified using nucleotide BLAST program. Bacterial DNA was extracted as mentioned above (3.3.3.2) and the gene encoding for Subtilosin and/or Subtilin

bacteriocin gene was amplified using primer *Sbo/Spa* PCR and run on 1.5% agarose gel stained with ethidium bromide. PCR was performed using Subtilin (*Spa*) and Subtilosin (*SboA*) (Sutyak et al., 2008) encoding primers (primer sequence as mentioned in table 3.1), in 50-µL reaction mixtures using 2 µL (10-50ng/µl) DNA, 0.1 mM of each set primers, 1.5mM $MgCl_2$, 0.1mM dNTPs mix and 2 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to run at an initial cycle of denaturation (94 °C for 2 min), followed by 34 cycles of denaturation (94 °C for 30 s), annealing (52.5 °C for 30 s) and elongation (65 °C for 1 min), ending with extension at 72°C for 7 min, in a DNA thermal cycler (BioRad T100^{TM} Singapore). The test was repeated in duplicate.

3.3.3.4 Gene expression assay

The isolates containing bacteriocin encoding gene were further evaluated for the gene expression under several cultivation conditions. The conditions for gene expression is a co-culture of isolates (mix/cocktail *S. aureus)* and *Bacillus* isolate in culture medium (nutrient broth) with varying salt concentration (5%, 10% & 15%). RNA was directly extracted from the culture following conventional RNA extraction method (Li et al., 2009) (Appendix A2). The purified RNA was converted to complementary DNA (cDNA) by reverse transcriptase reaction (Rio, 2014). The gene encoding for bacteriocin (subtilin and subtilosin) production was confirmed by PCR along with housekeeping gene BA-rpoB (F: 5'-GAC GAT CAT YTW GGA AAC CG-3'; R: 5'-GGN GTY TCR ATY GGA CAC AT-3') (Ko et al., 2004). The PCR amplicon was measured on 1.5% agarose gel electrophoresis respectively.

3.3.3.5 Laboratory method of performing gene expression:

i. Culture each strains of *S. aureus* (ATCC 25922, DMSc 6358, and food isolate 30-7) and *Bacillus* isolate in separate plate (NA+5%NaCl). Incubate at 37°C for 24 hours.
ii. Select single colony of each plate and inoculate in 2mL nutrient broth containing 3% NaCl. Incubate at 37°C for 24 hours and read the OD to 0.1 (10^{7-8} CFU/mL).

iii. Mix equal volume of different *S. aureus* strains (1:1:1) into a sterile tube to obtain cocktail *S. aureus.*

iv. Transfer 1mL of mixed *S. aureus* inoculum and 1ml of *Bacillus* isolate inoculum into nutrient broth (8mL) of various salt concentration (5%, 10% and 15%).

v. Incubate at 37°C for 24 hours.

vi. Centrifuge (12000rpm for 2 min) 1mL of each sample and wash the cells 2 times with sterile distilled water.

vii. Proceed with RNA extractions.

3.3.4 Safety evaluation of selected isolates

The safety of the selected isolates with inhibitory effect on cocktail *S. aureus* and gene encoding bacteriocin production were evaluated for any potential production of allergen; such as biogenic amine, hemolysin and also tested for acute toxicity and cytotoxicity. Acute toxicity was tested using both *in vivo* (mouse model) and *in vitro* (cell lines) Caco2 and Vero cell line.

3.3.4.1 Biogenic amines

PCR was performed to investigate the production of the following biogenic amines following multiplex PCR assay;

i. Histamine
ii. Tyramine
iii. Putrescine

The bacterial DNA extracted was subjected to PCR using the specific primers of 0.1mM. Specific primer used are JV16HC (367bp) / JV17HC (Jeune et al., 1995) and HDC3 (435 bp) / HDC4 for histamine. TD2 (1100 bp) / TD5 (Coton et al., 2010), TDC1 / TDC 2 (Fernández et al., 2007) and TDC-F (825 bp) / TDC-R (De las Rivas et al., 2005) for tyramine. And PUT1-F (1440 bp) / PUT1-R and PUT2-F (624 bp) / PUT2-

R (De las Rivas et al., 2005) for putrescine. The PCR was performed in total volume of 50 µL with 1.5mM $MgCl_2$, 0.1mM dNTPs mix and 1 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to an initial cycle of denaturation (94 °C for 2 min), followed by 34 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 30s), ending with extension at 72°C for 7 min, in a DNA thermal cycler (BioRad T100^{TM} Singapore). The test was repeated with duplicate. The PCR amplicon was measured on 1.5% agarose gel electrophoresis comparing to positive controls.

3.3.4.2 Screening for hemolysin production

The screening for hemolytic activity was tested using blood agar. Overnight colony culture was spotted onto the Blood agar plate and incubated at 37°C for 24 hours. The zone of hemolysis, either α, β or λ hemolysis around each isolate spot was observed (Mukry et al., 2010). The test was repeated with duplicate.

3.3.4.3 Acute/Cytotoxicity screening

The toxicity of substances can be tested by (a) studying the accidental exposures to a substance (b) *in vitro* studies using cells/ cell lines (c) *in vivo* exposure on experimental animals (Parasuraman, 2011). Acute toxicity is conducted using mouse model at Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand. Cytotoxicity was tested on both Caco2 and Vero cell line at BIOTEC, Rangsit, Thailand. The samples (cell extract) was prepared following crude cell extraction method using organic acid (methanol) and then air drying (Saleem et al., 2002).

3.3.5 Application of isolates as protective culture for bio-control in salt added foods

Prior to application as bio-control agent in food, both MIC and MBC measured following Clinical Laboratory Standard Internationals (CLSI) guidelines. The broth micro dilution method was employed to determine the MIC/MBC of bacteriocin produced from pure culture of *Bacillus* species.

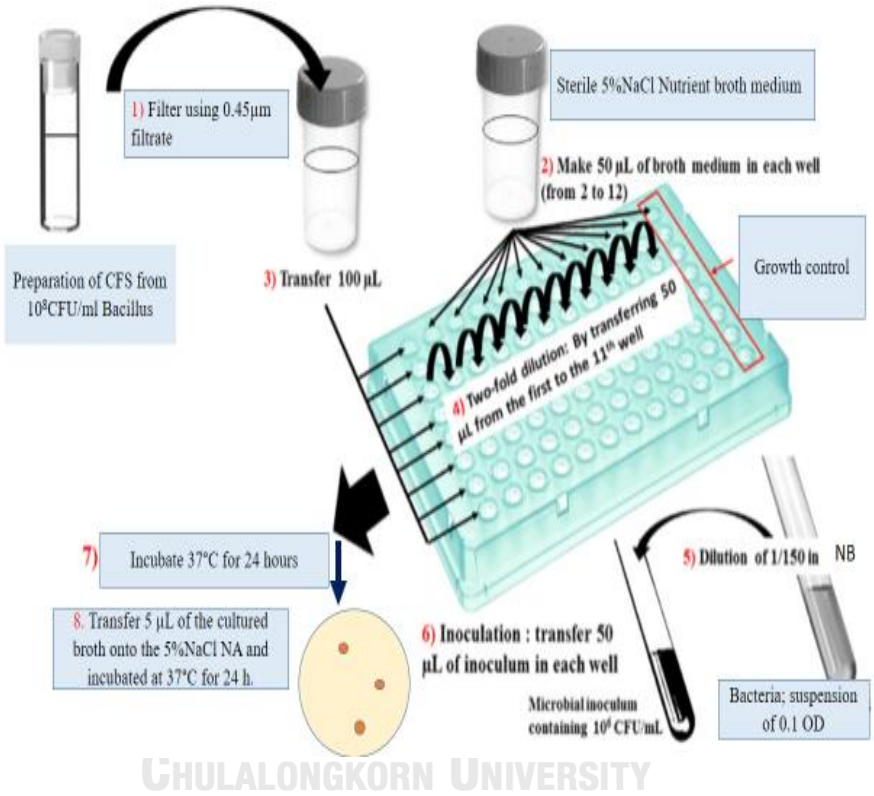


Figure 3.1 Broth micro dilution for antibacterial MIC/MBC testing as recommended by Clinical Laboratory Standard Internationals (CLSI) and Balouiri et al. (2016).

The bacterial culture with bacteriocin production property was initially grown in nutrient broth (5%NaCl) to log 8 and filtered using 0.45µm filtrate to collect cell free supernatant. 50µl of sterile nutrient broth was poured into each well. Then 100µL of CFS was added to the 1st well and then serially diluting by transferring 50µL from each well till the 11th well. 50µL of bacterial suspension (mixed *S. aureus* at 0.1OD) was added to all well except the control well. The microtitre plate was incubated for

24 hours at 37°C. After 24 hours the plate was read at 600nm and 5-10µL of cultured broth cultured on 5%NaCl+NA at 37°C, for 24 hours.

3.3.5.1 Protective culture preparation

Protective culture was prepared by direct inoculation of bacteriocin-producing *Bacillus*. Apart from production of bacteriocin, the culture also acts as protective culture, by eliminating the growth of other unwanted bacteria (Woraprayote et al., 2016). Two types of protective culture were prepared. Firstly, using skim milk powder and secondly, with freeze dried green mango pieces. Cell culture was obtained by growing bacterial culture mid stationary phase (OD 0.1 600 nm) in 5% NaCl NB, overnight at 37°C. Protective culture was prepared as follows, modified from Prakitchaiwattana et al., (2017) (a trade secret):

i. Cell culture obtained from mid stationary phase (10^{7-8} CFU/ml) nutrient broth supplemented with 5%NaCl.
ii. Take 5mL of broth and 6g of skimmed milk powder/freeze dried green mango pieces.
iii. Drying at 55°C for 30 minutes and then 50°C for 120 minutes, modified from (Rogers, 1914). Measure water activity until <0.6.
iv. Stored in sealed aluminum foil.
v. Determine the TPC following AOAC method.

The MIC/MBC of bacteriocin available in culture prepared and after mixing in food matrices were determined using the broth micro dilution method. This is to study if any deleterious effect of food matrix to the efficiency of bacteriocin produced by the *Bacillus* protective culture.

3.3.5.2 Food samples for application

Food samples for testing were collected and brought to laboratory from Bhutan, traditional Bhutanese cottage cheese (locally called as Datsi), shown in figure 3.2 (B), from Bumthang, and bamboo shoot pickle, figure 3.2 (A), locally prepared at Tsirang Bhutan were purchased from point of sale and transported to

laboratory for study purpose. Cottage cheese is produced as the action of acidification of milk. The food samples were handled aseptically. Cheese were stored at -20°C and pickle in room temperature until further study.

Figure 3.2 (Fig A) Bamboo shoot pickle. Fig (B)Bhutanese traditional cheese (Datsi)

The food samples (Bamboo shoot pickle and Bhutanese cottage cheese (Datsi)) were prepared in a portion of 50g cheese ball and pickle in bottle of 50 g gram 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. Initially, the food samples were tested for following parameters:

i. Total plate count - nutrient agar plate supplemented with 5%NaCl was plated with 0.1ml of sample and incubated at 37°C for 24 hours.
ii. Total Yeast and Mold count – potato dextrose agar supplemented with 10% tartaric acid was plated with 0.1mL of sample and incubated at 28°C for 5 days.
iii. pH – acidity of food sample was measured using pH meter, CyberScan® pH 1000 meter, Eutech instruments, Netherland.
iv. *Staphylococcus aureus* contamination – 1mL of sample was poured on Compact Dry X-SA and incubated at 37°C for 24 hours.
v. Salt (Martirani et al.) concentration – measured using standard curve, method mentioned by (Zhang and Xia, 2008).

The ready-to-use protective culture were prepared in skim milk powder and freeze dried green mango pieces as mentioned in 3.3.6. Prior to application into the food system MBC was studied and the amount of protective culture to be applied was calculated. One mg of protective culture was mixed to 9 grams of selected food sample, calculated from the amount of bacteriocin tested for MBC (being produced from log 8 of isolate culture) and final concentration derived using formula C1V1=C2V2. Finally the amount of protective culture applied was consider to be 10 times more than the MBC value as observed in section 3.3.5 (Leroy and De Vuyst, 2004, Holzapfel, 2002, Buckenhüskes, 1993) to be able to survive and grow due to the effect food matrixes.

The food samples mentioned above was divided into two portions with two replications. The first portion was mixed with the prepared protective culture and another was used as control treatment. The activity of protective culture to produce bacteriocin and its activity of was monitored during storage period (cheese stored at refrigerated temperature 4-6°C and bamboo shoot pickle at room temperature 26-27°C). TPC, *S. aureus* viability and pH was determined every 7 days, for 4 weeks (28 days) for bamboo shoot pickle and every 4 days, for 16 days for cottage cheese. Determination of the physical appearance, such as color changes, order and consistency was examined.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Halophilic bacteria from salty fermented foods

To study the microbial communities and isolation of Halophilic bacteria from salty fermented foods both the conventional cultural method and cultural independent method can be used. The study of microbial ecosystem in fermented food could help better understanding of the microbes present and their roles during fermentation. To study the microbial community, there are several improved methods and the use of culture dependent method is less effective, due to the lack of the knowledge of their natural environment, and difficulty in preparation of media resembling it. Therefore, molecular technique such as denaturing gradient and temperature gradient gel electrophoresis provide better result and understanding of actual bacterial community. In this study both, conventional culture method, using nutrient agar supplemented with 5% NaCl for isolating halophile bacteria and culture independent (Denaturing Gradient Gel Electrophoresis-DGGE) molecular method was used.

In this study, the molecular technique using Rev-T-PCR-DGGE targeting V3 region of 16s rRNA gene, using universal bacterial primers, firstly 27F/1492R (Frank et al., 2008) and second set of V3f with GC clamp-Uni-0515r was used to perform nested PCR. The amplicon was run on 8% denaturant gradient polyacrylamide gel to discriminate the cDNA bands from mixed microbial cells. It was found *Halanaerobium* spp. to be the main population expressed in Plara fermentation and were consistently present in all fermentation conditions, along with *Lactobacillus acidpiscis*, as presented in figure 4.2 and table 4.1. This cultural independent method demonstrated the key bacteria as *Halanaerobium* spp. which could play an important role in Plara fermentation. The detection of these bacteria in similar fermented conditions was previously reported and *Lactobacillus acidpiscis* was reported as main bacteria present in Plara ecosystem by Tanasupawat et al. (2000) but its role in the fermentation system was not mentioned. *Halanaerobium* was found as the predominant bacteria in saeu-jeot: traditional Korean salted seafood, in a study

conducted by Jung et al. (2013). However, for the later bacteria, there was some important information that could demonstrated their role in Plara fermentation. The study on *Halanaerobium* isolate genomes by Booker et al. (2017) from metagenomic data sets reconstruction genomes revealed the conserved presence of rhodanese-like proteins and anaerobic sulfite reductase complexes is capable of converting thiosulfate to sulfide and giving the fermented produce its unique taste, odor and also dark color as an unique color of Plara. Moreover, *Halanaerobium* could be responsible for production of metabolites; acetate, butyrate, and methylamines, which could be considered as a potential indicator to decide the appropriate fermentation time (Jung et al., 2013). Thus, the consistently presence of *Halanaerobium* throughout Plara fermentation and the properties of resulted Plara as observed in this study could help confirm its important role in salty fish fermentation.

In soya sauce, the results are shown in figure 4.1 and table 4.1. In current study, *Staphylococcus gallinarum* along with *Lactobacillus delbrueckii* were detected as the predominant bacterial spp. present in soya sauce ecosystem. This results corresponded to (Tanaka et al., 2012). They demonstrated that apart from the yeast, such as, *Aspergillus oryzae*, *Zygosaccharacharocyces rouxii*, *Candida* spp. found as main microbes, the bacteria's, such as *Bacillus* spp., *Staphylococcus gallinarum* and occasionally *Lactobacillus* spp. were also present in the system and influenced on soya fermentation. *Staphylococcus gallinarum* is continuously detected in soya sauce due to its high number of viable cells and play an important role during fermentation, including inhibiting the growth of some pathogenic bacteria. Importantly, *Staphylococcus gallinarium* produces a lantibiotic bacteriocin known as gallidermin (Kellner et al., 1988).

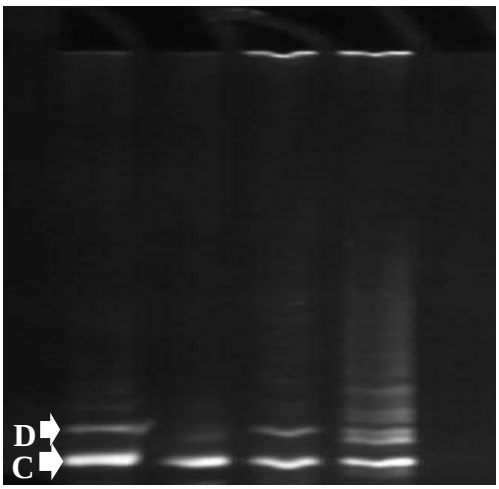


Figure 4.1 Denaturing gradient gel electrophoresis (DGGE) profiles of PCR-amplified cDNA of Soya sauce (SS) samples.

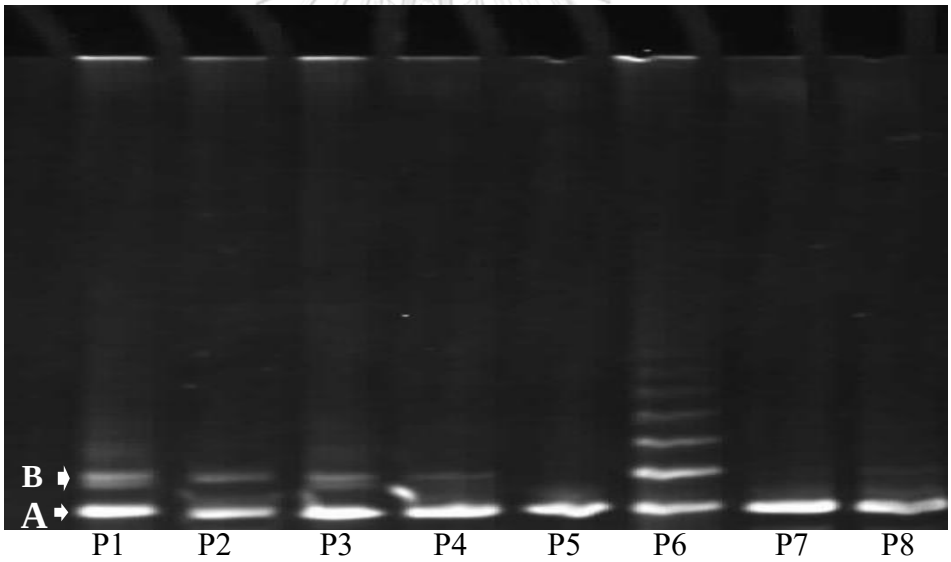


Figure 4.2 Denaturing gradient gel electrophoresis (DGGE) profiles of PCR-amplified cDNA of Plara (P) samples

Table 4.1 Identity of bands.

Obtained from denaturing gradient gel electrophoresis (DGGE) analysis of figure 4.1 & 4.2 after nucleotide sequencing analysis using BLAST program

Band[a]	**Species**	**Identification homology (%)**	**Accession number** [b]
A	Uncultured *Halanaerobium* spp.	98	KU961746.1
B	*Lactobacillus acidpiscis*	98	KX139191.1
C	*Staphylococcus gallinarum*	98	MF399027.1
D	*Lactobacillus delbrueckii*	95	MF446929.1

[a]Bands as marked on DGGE gel

[b]Accession number of the sequence and identification percentage of the closest relative fond by BLAST search (www.ncbi.nlm.nih.gov/)

For cultural plating method, nutrient agar supplemented with 5% NaCl was used to enumerate and isolate microbial from Plara and soya sauces. The total number of population in Plara samples varied from 10^2- 10^8 CFU/g as shown in table 4.2, which is similar to the finding of Tanasupawat et al. (1998). The types of colony observed on the plate were not diverse, with only two to three types of colonies isolated, as shown in table 4.2. The representative colonies were picked by Harrison disc method based on colony morphology. The main isolates found were closed to *Bacillus* and the other groups occasionally found were *Staphylococcus* group, (results confirmed after sequencing analysis). Few pigment producing bacteria were also observed. The isolates were Gram stained and view under oil immersion. It was observed that the isolates were mainly Gram positive, short chain with spores. Based on cell morphology and Gram staining and spore forming characteristic, these

isolations were close to *Bacillus* spp. This could be supported by the report of Tanasupawat et al. (2000) demonstrating that bacteria generally found in Plara were *Bacillus*, such as *B. subtilis* and *B. licheniformis*, and other halotolerant bacteria, particularly, *Staphylococcus* spp.

Though *Halanaerobium* and *Staphylococcus gallinarum* were detected as the predominant bacteria by culture independent Rev-T-PCR-DGGE method, which otherwise were not isolated by cultural method. This could be because, *Halanaerobium* is an obligate anaerobe which could not be grown under the cultural condition conducted in this study. Where else, minor population existing bacteria, such as *Bacillus*, on contrary was observed as main isolates on the agar media. *Bacillus* spp. is spore forming, Gram positive bacteria growing under aerobic condition and generating hydrogen sulfide during metabolic conversion of methionine to cysteine (Kadota and Ishida, 1972). Thus, hydrogen sulfide could play a major role in changes of Plara properties during fermentation adding its distinctive odor and dark color.

Interestingly, it was also noticed that, those Plara samples that harbored colony-like *Staphylococcus* spp. had low count of colony-like *Bacillus* Spp. Contrarily, the samples that harbored *Bacillus* spp. had low *Staphylococcus* spp. or completely absent. This indicated the buried relationship between this two bacterial groups and suggested their possible antagonism mechanisms. Moreover, *Bacillus* spp. are known to produce various bacteriocin or bacteriocin like inhibitory substances compounds (Smitha and Bhat, 2013). Thus, this information could help us predict the inter-relation among the different species.

Finally, the total of 124 halophile isolates, 22 isolates from four soya sauce and 102 isolates form 17 Plara samples were isolated. All of this isolated were subjected for salt tolerance testing and study the possibility of bacteriocin or BLIS production.

Table 4.2 Comparison of culture dependent and culture independent results

Sample	Period of fermentation	Culture dependent			Culture independent DGGE Result	NaCl Conc. (%)
		Total plate count CFU/ml	Isolate number (Herisson disc)	Isolate identity (%)		
SS1	Final product	2.09×10^{5}	8	Unidentified organism SS1 (100%)	*Staphylococcus gallinarum* *Lactobacillus delbrueckii*	19.37
SS2	Final product	7.20×10^{7}	13	*B. subtilis* (100%)	*Staphylococcus gallinarum* *Lactobacillus delbrueckii*	17.29
SS3	4-month	6.35×10^{4}	7	Unidentified organism SS3A (70%) Unidentified organism SS3B (30%)	*Staphylococcus gallinarum* *Lactobacillus delbrueckii*	18.53
SS4	4-month	2.90×10^{2}	3	Unidentified organism SS4 (100%)	*Staphylococcus gallinarum* *Lactobacillus delbrueckii*	17.34
P1	Final product	7.70×10^{3}	15	*B. subtilis* (66%) *B. pumilus* (34%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	25.63
P2	Final product	5.60×10^{3}	7	*B. pumilus* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	21.85
P3	1-month	1.83×10^{6}	12	*B. subtilis* (50%) *S. epiderdimis* (50%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	20

P 4	Final product	1.83×10^{8}	18	*B. subtilis* (50%) *B. atrophaeus* (50%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	31.11
P 5	Final product	2.5×10^{8}	20	*B. subtilis* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	31.70
P 6	Final product	2.82×10^{4}	40	*B. subtilis* (50%) *B. amylilquefaciens* (25%) *B. licheniformis* (12.5%) *Lelliottia* (12.5%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	20
P 7	4-month	7.00×10^{6}	7	*B. licheniformis* (66%) *B. pumilus* (34%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	31.56
P 8	Final product	4.20×10^{7}	11	*B. subtilis* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	34.01
P 9	Final product	4.60×10^{4}	6	*B. amyloliquefaciens* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	22
P 10	Final product	2.84×10^{4}	20	*B. subtilis* (50%) *B. licheniformis* (50%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	24.65
P 11	Final product	9.30×10^{7}	17	*B. pumilus* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	23.62

P12	Final Product	1.30×10^{3}	13	*B. pumilus* (66%) *B. atropaheus* (34%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	24.90
P13	Final product	1.64×10^{6}	28	*B. pumilus* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	8.45
P14	Final product	4.5×10^{4}	20	*B. pumilus* (50%) *B. altitudinis* (50%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	17.55
P15	Final product	2.02×10^{4}	20	*B. subtilis* (50%) *B. amyloliquefaciens* (50%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	25.61
P16	Final product	2.40×10^{5}	24	*B. subtilsi* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	22
P17	Final product	9.00×10^{2}	8	*B. amyloliquefaciens* (100%)	Uncultured *Halanaerobium* spp *Lactobacillus acidpiscis*	21.29

There are some reports that demonstrated the Thai fermented fish (Plara) and soya sauce contained high concentrations of NaCl, 11.5 – 23.5% and low pH 4.3 – 5.6 (Tanasupawat et al., 1998). However, salt concentration in samples collected from various production areas in this study were relatively different. NaCl concentration of soya sauce was found to be 17-19% and in Plara it varied, ranging from 8-34% (Table 2.4). The bacterial strain isolates from this kinds of salty products as shown in the Table 2.4 are mostly halophile bacteria. In salt containing foods, although, the high concentration of NaCl controls the growth of many bacteria; as

most of the bacteria, including both pathogenic and spoilage can rarely survive this high salt condition (Tanasupawat et al., 1993). Under this condition, there are some useful halophilic bacteria that can survive well in very high salt concentrations. Since this study aimed to isolate such halophilic bacteria having inhibitory effect on other halophilic pathogens, especially, *Staphylococcus aureus* and to be applied as bio-control agent in salt containing or added foods. The bacterial ability to grow in various salt concentrations was one of the important criterion to select the protective culture. As shown in the figure 4.3, it was found that almost all (100%) of the isolates could grow well in cultural media containing 1% and 5% NaCl. The isolates of up to 93%, 82% and 78% could still grow well in 10%, 15% and 20% NaCl, respectively. Thus, the isolates obtained from both Plara and soya sauce were mainly highly halophilic bacteria, which shows growth under the salt concentration over 5% (Thongsanit et al., 2002).

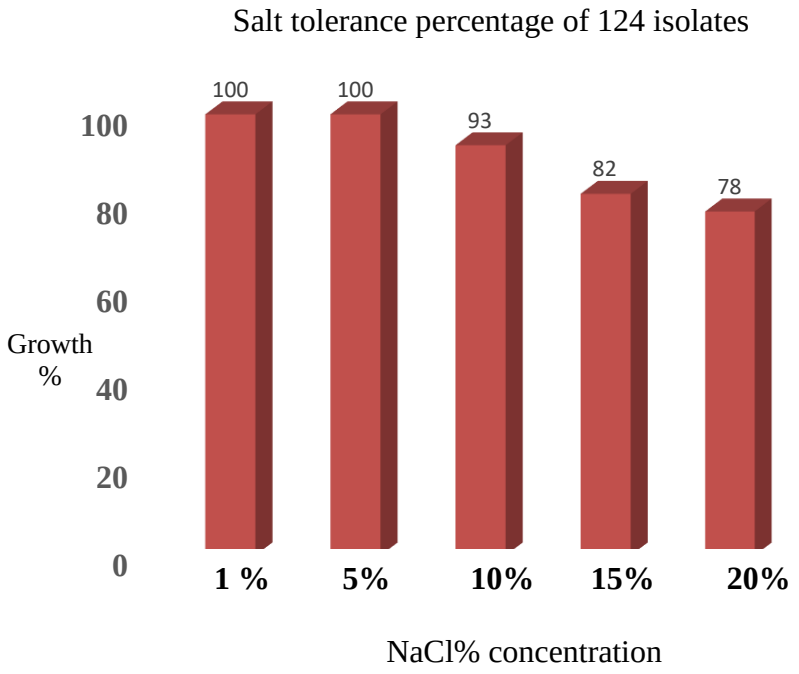


Figure 4.3 Ability of bacterial isolates growth in different salt concentrations

To investigate the factors associated to the presence of bacterial in the food systems, the relationship with salt concentrations, duration of fermentations and total plate counts (TPC) was then drawn. In the soya sauces, since the salt concentrations of all samples were relatively similar and the TPC were also observed at the similar levels, this could not clearly demonstrate any effect of salt concentration on the bacteria community. In the Plara samples, although the TPC of all samples were significant diverse (ranged from 10^2CFU/mL to 10^8CFU/mL), the direction of the correlations still could not be predicted. However, there are several factors that could influence on the microbial community including making process and particularly, geographical factors which is reported as the main factor influencing on the prevalence of microbial strains in the ecosystems of indigenous fermented foods. Thus, to clarify this point, phylogenetic tree of bacteria DNA of all isolated strains was then constructed (Fig. 4.5) and also to find the relationship among bacterial isolates, associated factors and subjected to be discussed in the next section.

4.2 Bacteriocin producer and identification

4.2.1 Screening of Bacteriocin producer

In this study *Staphylococcus* species were occasionally found with relatively lower population in Plara samples. This observation showed that, *Staphylococcus* group despite being halotolerant species, seemed to be controlled by some factors. This could be due to the antagonistic effect of the competitor strains or some inhibitory agents in the system, such as bacteriocin production from other bacteria. Therefore, the halophilic bacteria isolated from the cultural plating were investigated for any bacteriocin production property on target bacteria. The inhibitory effect was assayed in term of zone of inhibition (ZoI) by following spot on lawn culture. The inhibitory activity of all 124 isolated were preliminarily screened (data not shown) and positive strains were then subjected to identify by 16S rDNA sequencing analysis.

The bacterial DNA of isolates with inhibitory activity during screening was harvested and amplified using universal bacterial primers (338F/518R). Three types of ribosomal RNA are present in bacterial cells; one small ribosomal subunit (16S

rRNA) and two large ribosomal subunit (5S and 23S rRNA) (Hunt et al., 2006). The highly conserved region is present in 16S rRNA-gene-sequence in almost all bacterial species (Dorn-In et al., 2015). The appendix D7 shows the presence of bacterial gene with approximately 193bp (Frank et al., 2008) checked before sending for sequencing.

The sequencing results were analyzed using nucleotide Basic Local Alignment Search Tool (nBLAST) and phylogenetic tree constructed using Geneious program. This sequencing analysis will allow the differentiation of PCR products (Schmalenberger et al., 2001). From the sequencing results, most of the isolates were identified to be *Bacillus* bacteria, with predominance of *Bacillus subtilis* (41%), followed by *Bacillus pumilus* (19%), *Bacillus amyloliquefaciens* (16%), *Bacillus licheniformis* (11%), *Bacillus atrophaeus* (5%) and occasionally *Bacillus altitudies* (3%) and also some other bacteria including, *Staphylococcus* species 5%, that were identified with positive inhibitory action. The percentage of different bacterial species positive with inhibitory action and identified after sequencing analysis is shown in the figure 4.4.

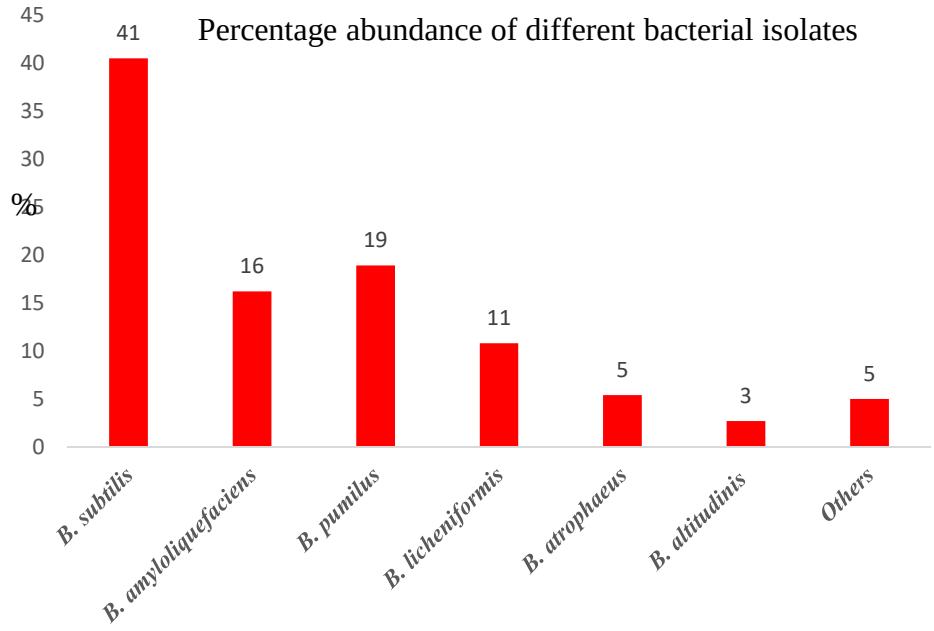


Figure 4.4 Percent of different bacterial isolates (result of nucleotide BLAST sequencing analysis)

The study on production of bacteriocin or BLIS by the *Bacillus* species were conducted following deferred antagonism method (spot-lawn culture technique), as shown in the Appendix D4. Interesting, the results revealed diverse inhibitory pattern, as will be discussed below:

All of the six different strains of *Bacillus* (*B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. atrophaeus* and *B. altitudies)* isolates exhibited inhibitory effect on target organisms but with different pattern. The inhibitory activities of these strains could possibly be an action of bacteriocin encoding by specific gene in their genomes since many species of *Bacillus* are known to produce bacteriocin and their genetic mechanisms had been well studied. The important bacteriocin such as subtilin, and subtilosin is produced by *B. subtilis* and other closely related *Bacillus* species (Chan et al. (1993).

In the *current* study, *B. subtilis* was found as the predominance (41%) species and had inhibitory action on all the *S. aureus* strains, including cocktail *S. aureus*, as shown in table 4.3. *Bacillus subtilis* was isolated from both the samples; soya sauce (SS), and Plara (P) samples. Almost all the isolates of *B. subtilis* exhibited similar range of zone of inhibition, except for the few isolates, isolate P6-1 and P6-9 demonstrated significantly higher ($p<0.05$) zone of inhibition to all the strains of target *S. aureus*. On the other hand, isolate P6-12 demonstrated inhibitory action only on food grade *S. aureus* strain. This differences could be due to the maturity of the produced bacteriocin (Velho et al., 2013, Kawulka et al., 2004). Although the isolates P6-1, P6-2, P6-9 and P6-12 (table 3.1) were isolated from the same Plara sample, they produced different inhibitory action. This could reflect the non-influence of geographical factor on phenotype and/or genotype properties of microorganism existing in the Plara ecosystems.

Table 4.3 Zone of inhibition (in mm) of *B. subtilis* on different strains of *S. aureus*

Isolate No.	*Isolate spp.*	ATCC 25922	DMSc 5358	Food isolate *S. aureus*	Cocktail *S. aureus*
P6-1	*B. subtilis*	0.60 ± 0.10^{fghij}	0.73 ± 0.06^{gh}	0.70 ± 0.10^{fg}	0.60 ± 0.20^{fgh}
SS2-5	*B. subtilis*	0.53 ± 0.12^{defgh}	0.53 ± 0.06^{cdefg}	0.63 ± 0.06^{efg}	0.43 ± 0.06^{def}
P3-11	*B. subtilis*	0.33 ± 0.06^{bcdef}	0.53 ± 0.12^{cdefg}	0.47 ± 0.12^{cdef}	0.53 ± 0.12^{efgh}
P16-1	*B. subtilis*	0.27 ± 0.03^{bcd}	0.40 ± 0.00^{bcde}	0.00^{a}	0.50 ± 0.10^{defgh}
P10-3	*B. subtilis*	0.40 ± 0.00^{bcdefg}	0.37 ± 0.06^{bcd}	0.47 ± 0.12^{cdef}	0.30 ± 0.00^{bcd}
P6-2	*B. subtilis*	0.33 ± 0.06^{bcdef}	0.33 ± 0.06^{bcd}	0.00^{a}	0.37 ± 0.06^{bcde}
P6-12	*B. subtilis*	0.00^{a}	0.50 ± 0.10^{bcdefg}	0.00^{a}	0.00^{a}
P4-9	*B. subtilis*	0.83 ± 0.15^{j}	0.87 ± 0.12^{h}	0.73 ± 0.12^{fg}	0.37 ± 0.06^{bcde}
P8-1	*B. subtilis*	0.53 ± 0.12^{defgh}	0.47 ± 0.06^{bcdefg}	0.33 ± 0.11^{bcd}	0.37 ± 0.06^{bcde}
P8-2	*B. subtilis*	0.37 ± 0.06^{bcdefg}	0.43 ± 0.15^{bcdef}	0.53 ± 0.12^{cdef}	0.23 ± 0.11^{bc}
P1-3	*B. subtilis*	0.37 ± 0.15^{bcdefg}	0.47 ± 0.31^{bcdefg}	0.00^{a}	0.30 ± 0.00^{bcd}
P6-9	*B. subtilis*	0.80 ± 0.20^{ij}	0.87 ± 0.12^{h}	0.83 ± 0.12^{g}	0.40 ± 0.00^{cde}
P5-6	*B. subtilis*	0.73 ± 0.12^{hij}	0.70 ± 0.10^{fgh}	1.07 ± 0.12^{h}	0.67 ± 0.06
P1-4	*B. subtilis*	0.47 ± 0.12^{cdefgh}	0.53 ± 0.12^{cdefg}	0.00^{a}	0.00^{a}
P15-1	*B. subtilis*	0.60 ± 0.20^{fghij}	0.27 ± 0.03^{bc}	0.33 ± 0.06^{bcd}	0.00^{a}

Note: Different lower-case superscripts within a column indicate significant difference ($p<0.05$)
Values are mean ± standard deviation of triplicate sample determination

Bacillus amyloliquefaciens is the common group of *Bacillus* species isolated from fermented food products. Though *B. amyloliquefaciens* demonstrated inhibitory action on the target *S. aureus* but the results were not consistence. For instance, the isolate number P6-11 and P9-1 did not show inhibitory action on DMSc *S. aureus* strain. Similarly, the isolates P6-5/P6-11, and P9-2/P9-1 though was isolated from similar source but demonstrated different inhibitory action, indicating non-influence

of geographical factor. *Bacillus amyloliquefaciens* strains is also a potential bacteriocin producer and their bacteriocin production properties has been already reported in some literatures. Kaewklom et al. (2013) isolated *B. amyloliquefaciens* from Thai fish paste (Kapi) and known to produce bacteriocin active against halotolerant pathogen *L. monocytogenes.* In the next few years, Lim et al. (2016) in their study isolated *B. amyloliquefaciens* from soil and found it active against *S. aureus* and some Gram negative bacteria. Hence, the inhibitory action of *B. amyloliquefaciens* in current study could be due to the action of bacteriocin or BLIS produced by this group of halophile bacteria. Range of activity zone of the various strains of *B. amyloliquefaciens* isolates are shown in table 4.4.

Table 4.4 Zone of inhibition (in mm) of *B. amyloliquefaciens* on different strains of *S. aureus*

Isola No.	*Isolate spp.*	ATCC 25922	DMSc 5358	Food isolate *S. aureus*	Cocktail *S. aureus*
P6-5	*B. amyloliquefaciens*	0.57 ± 0.06^{bcdef}	0.37 ± 0.06^{bc}	0.40 ± 0.00^{bc}	0.43 ± 0.06^{efgh}
P6-11	*B. amyloliquefaciens*	0.20 ± 0.10^{abc}	0.00^{a}	0.30 ± 0.00^{bc}	0.43 ± 0.06^{def}
P9-2	*B. amyloliquefaciens*	0.27 ± 0.06^{bcd}	0.43 ± 0.15^{bcdef}	0.00^{a}	0.00^{a}
P9-1	*B. amyloliquefaciens*	0.20 ± 0.10^{abc}	0.00^{a}	0.20 ± 0.10^{ab}	0.43 ± 0.06^{def}
P15-2	*B. amyloliquefaciens*	0.57 ± 0.06^{efghi}	0.47 ± 0.15^{bcdefg}	0.47 ± 0.40^{cdef}	0.53 ± 0.06^{efgh}
P17-1	*B. amyloliquefaciens*	0.57 ± 0.06^{efghi}	0.37 ± 0.06^{bcd}	0.40 ± 0.00^{bcde}	0.43 ± 0.06^{def}

Note: Different lower-case superscripts within a column indicate significant difference (p<0.05). Values are mean ± standard deviation of triplicate sample determination.

Bacillus licheniformis isolates from Plara sample exhibited inhibitory action on *S. aureus* but the inhibitory zone size was relatively low. A strain of *B. licheniformis* P7-1, as shown in the table 4.5, could inhibit only ATCC strain of *S. aureus* and not the other strains of *S. aureus* probably because the efficacy of bacteriocin produced was low or immature. *Bacillus licheniformis* is widely spread in natural sources and known to produce many peptides with antibacterial activity (He et al., 2006). Bacitracin is the first peptide antibiotic derived from cultures of *B. licheniformis* (Johnson et al., 1945). This *Bacillus* strain produced compounds that showed strong inhibitory activity to *S. aureus* and *Salmonella enterica* ser (Guo et al. (2012). Similarly, Martirani et al. (2002) and He et al. (2006) isolated *B. licheniformis* from various food sources that demonstrated strong antimicrobial activity against Gram positive bacteria, including *S. aureus*. These reports support the possible mechanism of inhibitory action due to bacteriocin production.

Table 4.5 Zone of inhibition (in mm) of other Bacillus spp. and other bacterial isolates on different strains of *S. aureus*.

Isolate No.	*Isolate spp.*	ATCC 25922	DMSc 6358	Food isolate *S. aureus*	Cocktail *S. aureus*
P13-1	*B. pumilus*	0.47 ± 0.12^{cdefgh}	0.40 ± 0.00^{bcde}	0.00^{a}	0.33 ± 0.06^{bcde}
P14-2	*B. pumilus*	0.33 ± 0.06^{bcdef}	0.67 ± 0.12^{efgh}	0.60 ± 0.26^{defg}	0.67 ± 0.12^{h}
P12-3	*B. pumilus*	0.43 ± 0.15^{bcdefg}	0.70 ± 0.10^{fgh}	0.40 ± 0.00^{bcde}	0.47 ± 0.06^{defg}
P12-4	*B. pumilus*	0.30 ± 0.10^{bcde}	0.23 ± 0.10^{ab}	0.00^{a}	0.37 ± 0.10^{bcde}
P1-6	*B. pumilus*	0.43 ± 0.15^{bcdefg}	0.47 ± 0.12^{bcdefg}	0.50 ± 0.26^{cdef}	0.37 ± 0.06^{bcde}
P7-5	*B. pumilus*	0.37 ± 0.06^{bcdefg}	0.33 ± 0.06^{bcd}	0.00^{a}	0.33 ± 0.06^{bcde}
P11-1	*B. pumilus*	0.27 ± 0.10^{bcd}	0.47 ± 0.12^{bcdefg}	0.00^{a}	0.33 ± 0.06^{bcde}
P7-2	*B. licheniformis*	0.47 ± 0.12^{cdefgh}	0.37 ± 0.02^{bcd}	0.47 ± 0.12^{cdef}	0.20 ± 0.10^{b}
P10-7	*B. licheniformis*	0.27 ± 0.12^{bcd}	0.43 ± 0.15^{bcdef}	0.40 ± 0.17^{bcde}	0.33 ± 0.06^{bcde}

P6-6	*B. licheniformis*	0.17 ± 0.02^{ab}	0.23 ± 0.11^{ab}	0.20 ± 0.10^{ab}	0.00^{a}
P7-1	*B. licheniformis*	0.33 ± 0.06^{bcdef}	0.00^{a}	0.00^{a}	0.00^{a}
P12-1	*B. atrophaeus*	0.63 ± 0.06^{ghij}	0.40 ± 0.00^{bcde}	0.57 ± 0.15^{cdef}	0.53 ± 0.12^{h}
P4-2	*B. atrophaeus*	0.33 ± 0.06^{bcdef}	0.00^{a}	0.47 ± 0.21^{cdef}	0.30 ± 0.00^{bcd}
P14-1	*B. altitudies*	0.60 ± 0.20^{fghij}	0.57 ± 0.21^{defg}	0.57 ± 0.06^{cdef}	0.33 ± 0.06^{bcde}
P6-8	*Lelliottia*	0.60 ± 0.20^{fghij}	0.47 ± 0.12^{cdefg}	0.67 ± 0.12^{efg}	0.63 ± 0.06^{gh}
P3-1	*S. epidermidis*	0.47 ± 0.12^{cdefgh}	0.67 ± 0.12^{efgh}	0.57 ± 0.06^{cdef}	0.47 ± 0.12^{defg}

Note: Different lower-case superscripts within a column indicate significant difference ($p<0.05$). Values are mean ± standard deviation of triplicate sample determination.

In the current study few isolates of *B. atrophaeus* were isolated from Plara samples that demonstrated inhibitory action on the target organism. As shown in the table 4.5, the two strains of *B. atrophaeus* have different inhibitory action to each other. Though, this strain has not been intensively studied and limited report available on its bacteriocin production, the two researchers Ebrahimipour et al. (2014) and Shelar et al. (2012) mentioned its capacity of bacteriocin production and activity against many Gram positive bacteria, including *S. aureus.*

The other species is *B. pumilus* also capable of producing bacteriocin. A study in Thailand by Aunpad and Na-Bangchang (2007) isolated *B. pumilus* that produced active peptides that could inhibit *S. aureus*, including MRSA. Though the isolates of *B. pumilus* isolates in this study also demonstrated anti-*Staphylococcus aureus* activity but the results were not persistent and inhibited the food isolate *S. aureus* with least ZoI.

The other group of bacteria isolated during this study are *B. altitudines*, *Lelliottia* and *S. epidermidis.* There are not many studies that describe about the bacteriocin production by *B. altitudinis* but during this study an isolate of *B. altitudinis* (P14-1) was isolated that inhibited activity against all strains of *S. aureus*

used as target organism. *Staphylococcus epidermidis* are known to produce bacteriocin, such as epidermicin as reported by Sandiford and Upton (2012), active against many Gram positive bacteria but yet to be considered as food grade.

Overall, the *Bacillus* isolates respectively demonstrated inhibitory activity on target organism. It has been known that the *Bacillus* genes are closely related to each other and they probably transfer the gene encoding bacteriocin production through the mechanism of horizontal gene transfer (HGT) (Sutyak et al., 2008). Thus, the DNA nucleotide sequence of isolates were then subjected to construct phylogenetic tree, as shown in the figure 4.6. HGT mechanism relates the inhibitory action of *Bacillus* isolates from close and/or same locations as found in this study.

The *Bacillus* genus are known to have large ribosomal 16S diversity and can be isolated from various environments and includes both pathogenic and non-pathogenic strains. *Bacillus* isolates have a close evolution which could be divided into 3 different groups based on phylogenetic tree constructed by using Geneious program in current thesis, shown in Figure 4.5. The strains diversity seemed to have no relation to the source of isolate. This reflected that the geographical factor seemed to have no impact on the strain variety. On the other hand, it was found that the isolates with bacteriocin gene (investigated in the next section) were consistently obtained from high salt concentration Plara samples. This demonstrated the influence of salt concentration and salt could be acting as an inducer for bacteriocin expression. Again, from the phylogenetic tree, the strains containing subtilin gene (Fig 4.7) were located in the same cluster indicating that these strains had a very close genetic evolution (*B. subtilis* P15-1, *B. subtilis* P6-1, *B. subtilis* P5-6 and *B. subtilis* P6-12) whereas the other *B. subtilis* with no subtilin gene the direction in evolution of their 16S rDNA sequences were not found. The other isolates with only subtilosin gene (*B. subtilis* SS2-5) is located in another cluster and similarly an isolate of *B. atrophaeus* with subtilin was located in a separate cluster. This finding is relatively novel that need to further study whether the genetic evolution of the *Bacillus* group associated to the defensive mechanisms to survive in the natural habitats. This reflects the potential of this bacterial group to be developed as a bio-control agent for example in food manufacturing.

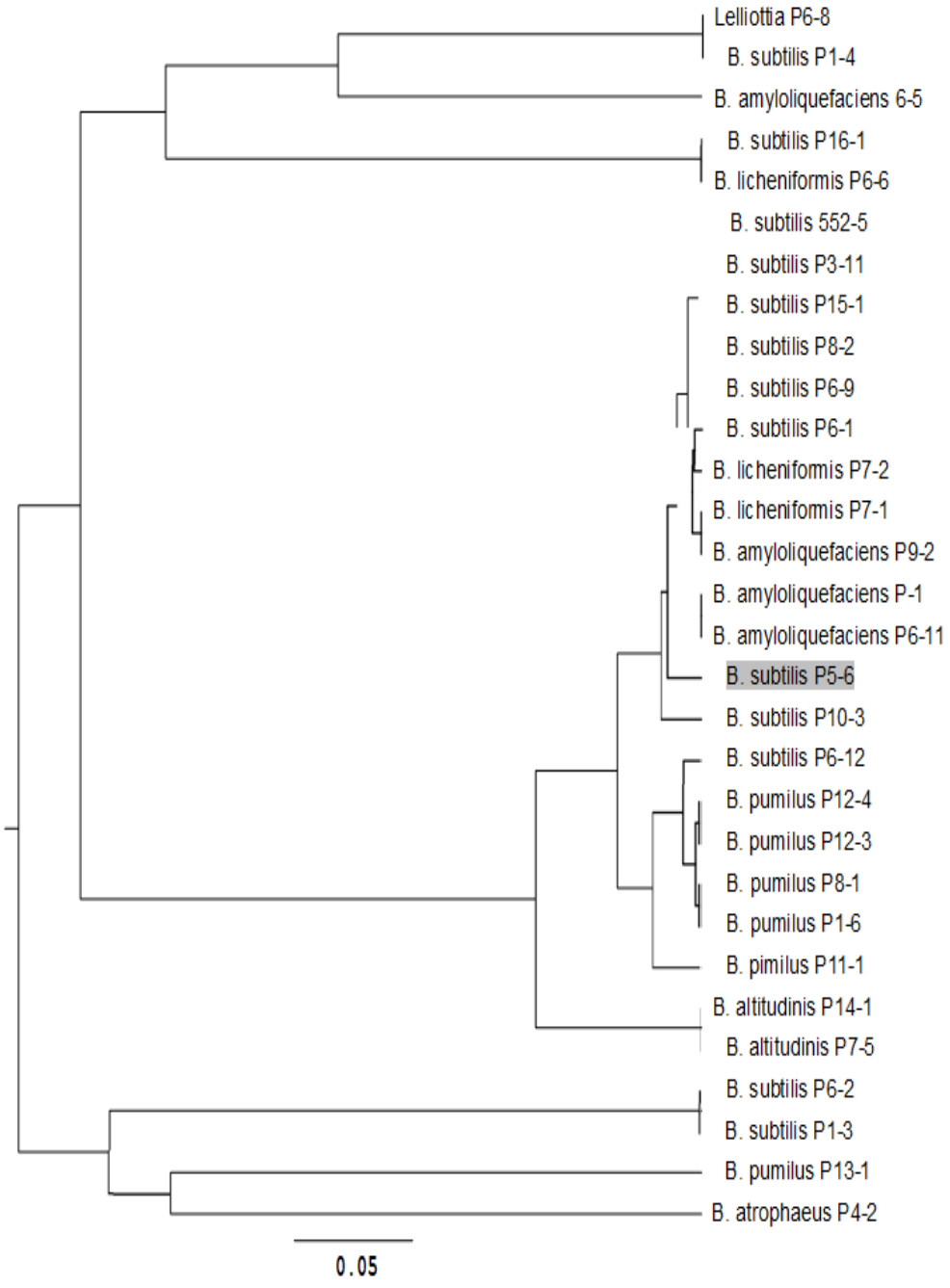


Figure 4.5 Phylogenetic tree for *Bacillus* species

Constructed using Geneious version 11.0 created by Biomatters.https://www.geneious.com

Based on inhibitory patterns, the *Bacillus* strains tested in this study inhibited food isolate *S. aureus* (FT30-7) and cocktail *S. aureus* culture with lowest inhibitory zone as compared to ATCC 25922 and DMSc 6538 strains, as shown in the figure 4.6. On the other hand, it would have shown similar inhibition on the mix culture to the single culture if it similarly inhibited all single strains tested. This differences could be due to the acquired immunity of different strains of *S. aureus* to protect their cells from toxins and/or bacteriocin by specific immunity gene or defensive mechanism as described by Zhu et al. (2014). Moreover, the food isolate *S. aureus* strain was isolated from Plara source and had been already exposed to the bacteriocin from *Bacillus*, this could be possibly due to the acquired immunity by this strain. Therefore, it can be concluded that ATCC 25922 strain of *S. aureus* being most sensitive strain and FT30-7 food isolate *S. aureus* as the least sensitive strain.

Percentage inhibition of different strains of *S. aureus* by *Bacillus* spp.

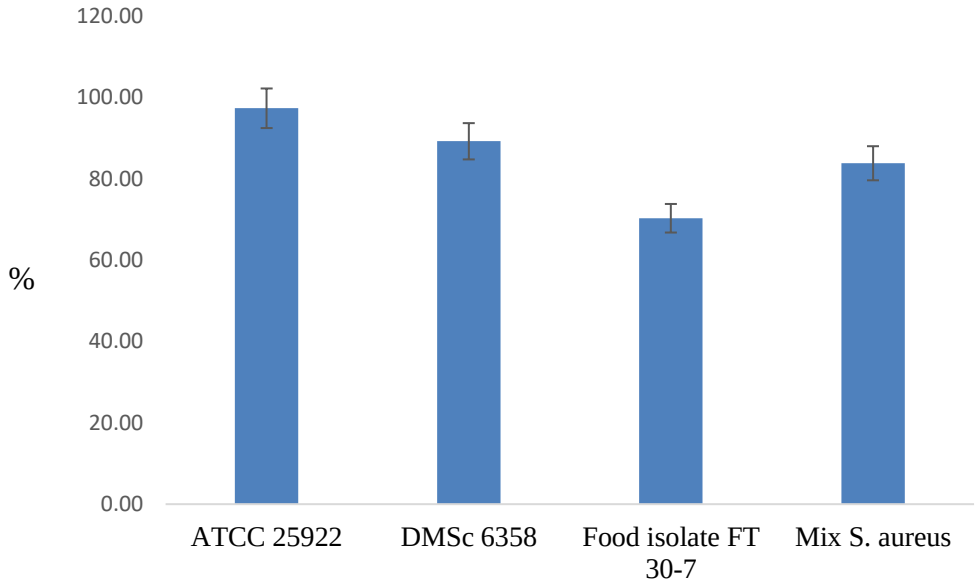


Figure 4.6 Percent inhibition of different strains of *S. aureus* by *Bacillus* bacteriocin

4.2.2 Gene encoding bacteriocin

The inhibitory action of *Bacillus* species on *S. aureus* with different zone of inhibition and pattern could potentially be due to the actions of some enzyme/protein(s) produced, especially, the production of bacteriocin or bacteriocin like inhibitory substances (BLIS) (Abriouel et al., 2010). The common bacteriocin produced by *Bacillus* spp. including their functional genes studied in parenthesis are subtilin (*spaS*) and subtilosin (*sboA*). It has been known that the *Bacillus* genes are closely related to each other and they probably transfer the gene encoding bacteriocin production through the mechanism of horizontal gene transfer (Sutyak et al., 2008). These gene transfer mechanisms, can occur via any of the three process such as, conjugation, transduction, or transformation (Rossi et al., 2014).

There were some previous reports demonstrated the bacteriocin production of *Bacillus* strains. Subtilosin and subtilin is produced by *B. subtilis* and as well as *B. amyloliquefaciens, B. licheniformis, B. atrophaeus* and other *Bacillus* strains (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 4.7, shows that among the 37 isolates with inhibitory action on target organism, only 9 isolates (24.30%) were positive for gene encoding either subtilin (size 566bp) or subtilosin (size 876bp), both or some other BLIS. Furthermore, 18% had gene encoding for only subtilin, 5.4% for only subtilosin gene and also 5.5% had both subtilin and subtilosin gene. Thus, indicating that subtilin was the major type of bacteriocin produced by *Bacillus* species isolates in this study.

An isolate of *B. subtilis* (P5-6) presented with both the gene encoding, subtilin and subtilosin (566bp and 876 bp respectively). It also exhibited significantly larger ($p<0.05$) inhibitory action zone relative to the other strains. An isolate of *B. subtilis* (P6-1) showed intense subtilin band on agarose gel (figure 4.7) and it was also a strain that exhibited significantly larger zone of inhibition. Interesting two isolates (P11-1 and P6-12) which had inhibitory action, but the DNA band observed on the gel were totally different from both subtilin and subtilosin. Their bands sizes were approximately 1000bp which was unable to identify. This demonstrated that

inhibitory properties of these two strains might be the action of other mechanisms, for example, bacteriocin encoding from the other gene, the BLIS and even common metabolizes.

There was not much difference in the pattern of zone of inhibition among the strains that were positive for gene encoding subtilin/subtilosin and those that did not show the presence of subtilin/subtilosin gene. This could probably be because there are other several different bacteriocin produced by *Bacillus* spp. (Chalasani et al., 2015) not include in the current study. For instance, *B. subtilis* (isolate P4-9) though it produced significantly higher ($p<0.05$) zone of inhibition but neither of these two genes, subtilin or subtilosin were present. But as mentioned earlier, *B. subtilis* are known to produce dozens of bacteriocin or BLIS, the inhibitory effect of isolate P4-9 could be potentially related to the other types of bacteriocin produced. Thus indicating that, there are other group of active bacteriocin being produced. Therefore, the isolates were independent to type of bacteriocin that they produced.

Finally, the isolate (P5-6) that showed the presence of both subtilin and subtilosin gene, exhibited consistent and significantly higher ($p<0.05$) inhibitory zone on the target organism. Moreover, the presence of double bacteriocin gene could be an added value of the organism. Therefore, the isolates with positive gene indication were selected for further gene expression experiments under different salt concentration and mixed *S. aureus* co-culture.

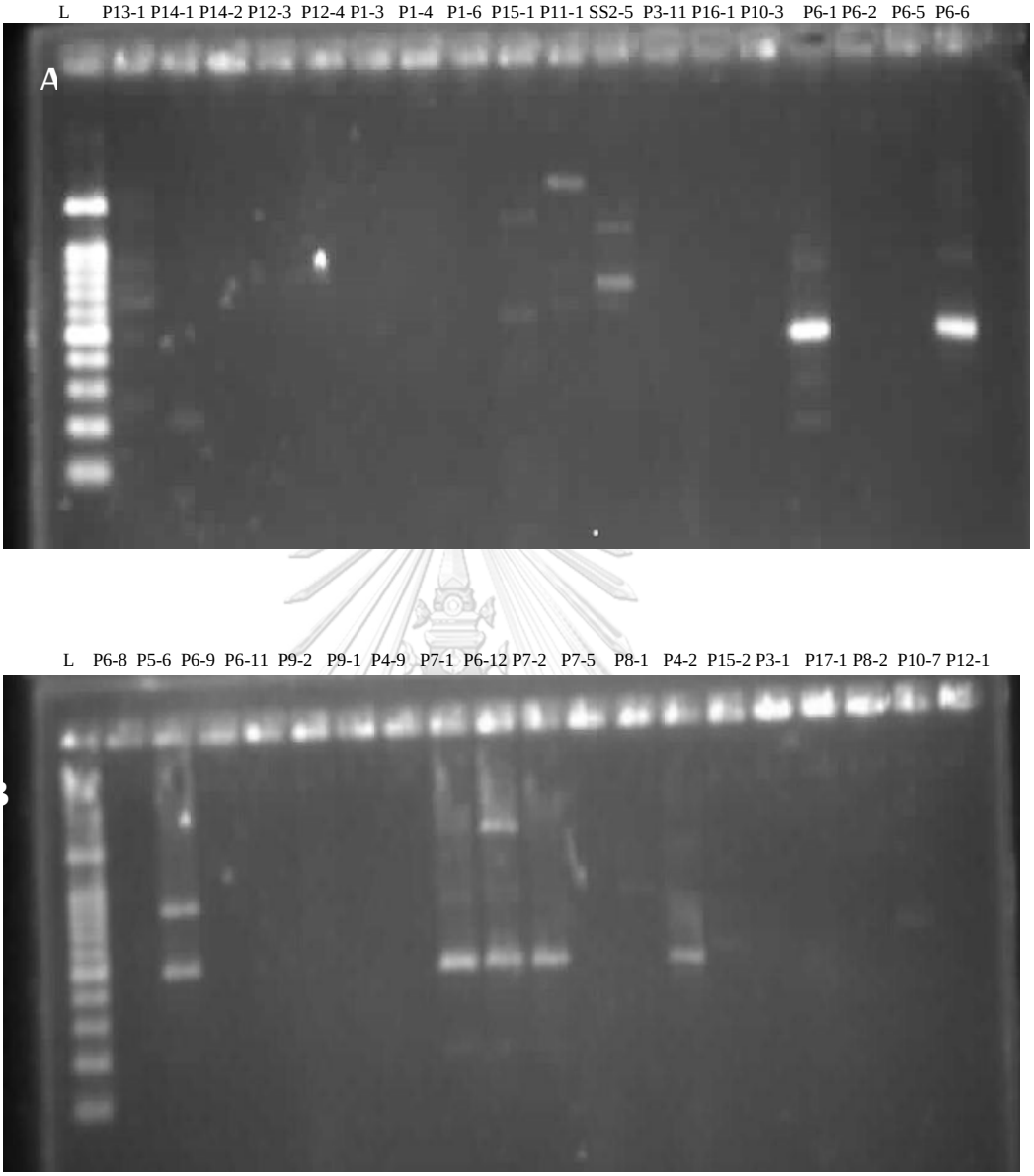


Figure 4.7A & B 1.5% agarose gel PCR product of bacteria for subtilin (566bp)/subtilosin (876bp) bacteriocin.

Table 4.6 *Bacillus* isolates encoding bacteriocin gene and their inhibitory zone

Sample no	Isolate spp	Zone of Inhibition				Potential type of bacteriocin		
		ATCC	DMSc	Food isolate	Mix	Subtilin	Subtilosin	unknown
P6-12	*B. subtilis*	-	+++	-	-	√		√
P5-6	*B. subtilis*	+++	+++	+++	+++	√	√	
P7-2	*B. licheniformis*	++	++	++	+	√		
P4-2	*B. atrophaeus*	++	-	++	+	√		
P15-1	*B. subtilis*	+++	+	++	-	√		
P6-1	*B. subtilis*	+++	+++	+++	+++	√		
P6-6	*B. licheniformis*	-	+	+	-	√		
SS2-5	*B. subtilis*	+++	+++	+++	++	-	√	
P11-1	*B. pumilus*	+	+++	-	++	-		√

Note: Zone of inhibition grading = **-** (size <0.9mm) insignificant inhibition, + (1-3mm) low inhibitory activity, ++ (size 3.1-4.9mm) moderate inhibitory activity, +++ (size >5.0mm) strong inhibitory activity. (√**)** gene present

4.2.3 Gene expression

Due to diverse pattern of inhibitory action and mixed detection of subtilin or subtilosin gene, gene expression is essential to rule out for selection of isolates for further study. Since, some isolates presented with bacteriocin gene but low inhibitory action (P6-6), and on the other hand, some isolates exhibited large inhibitory action (P6-9, P4-9) but no bacteriocin encoding gene detected. Therefore, to confirm the action due to bacteriocin expression and to be used in the food system deemed necessary. The bacteriocin encoding gene was evaluated for the factors that induce gene expression under different salt concentration conditions (5%/10% and 15% NaCl) 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).

Fig. A

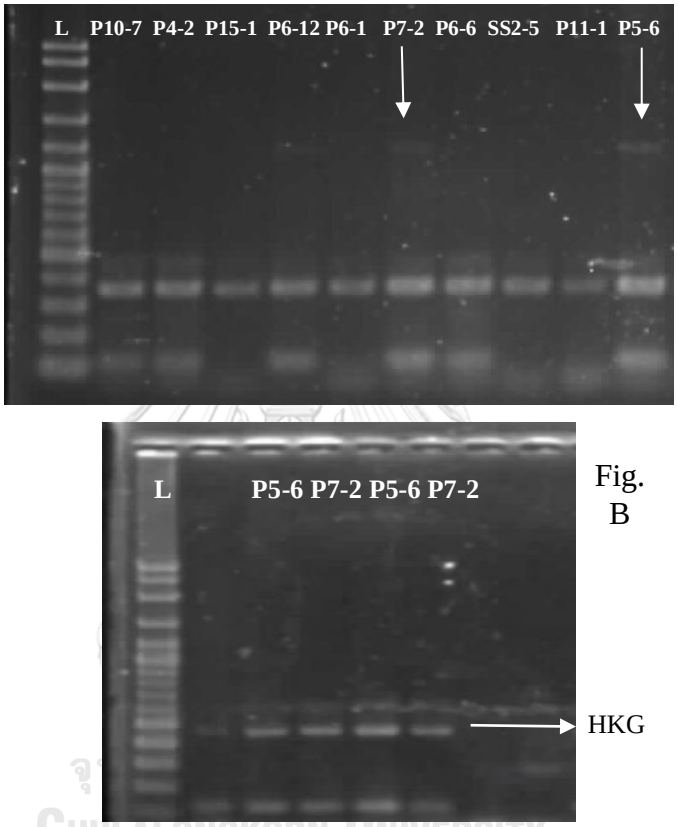


Figure 4.8 Gene expression study on 1.5% agarose gel electrophoresis.

Fig A. Screening of Gene expression using 5% NaCl and *S. aureus* co-culture with HKG. Two isolates P7-2 and P5-6 showed slightly positive bands due to over gene expression.

Fig B. Gene expression in 10%/15% NaCl and *S. aureus* with HKG. None of the isolates presented any visible bands when the salt concentration was increased to 10/15%.

Production of secondary metabolites from the *Bacillus* species, including *B. subtilis, B. amyloliquefaciens* and *B. licheniformis* were thought to be under complex genetic regulation (Lisboa et al., 2006). While screening for gene expression of *Bacillus* isolates inoculated along with cocktail *S. aureus* co-culture in 5% NaCl nutrient broth, two isolates; *B. subtilis* (P5-6) and *B. licheniformis* (P7-2) were detected for gene over expression with low band intensity on agarose gel, but when the salt concentration was increased to 10 and 15% neither of the band were detected. This could be due to the short gene expression period of bacterial cell and the signal disappeared after 5-6 hours, so could not be detected later than 6h with normal PCR protocol, as mentioned by (Stein et al., 2002), were else currently in 10-15%NaCl, it was measured after 48 hours. Moreover, the effect of higher salt concentration as suggested by Bhunia et al. (1991) could inhibit bacteriocin expression due to interference with the ionic interactions to the bacteriocin binding on the target cell leading to formation of NaCl-induced conformation changes in the peptide structure (Renye and Somkuti, 2015).

Table 4.7 Zone of inhibition of Cell free supernatant (CFS) obtained from gene expression experiment with 5%NaCl and *S. aureus* co-culture

Isolate Code	Bacterial spp.	Zone of Inhibition of CFS on mix *S. aureus*
P10-7	*B. licheniformis*	-
P4-2	*B. atrophaeus*	-
P15-1	*B. subtilis*	-
P6-12	*B. subtilis*	-
P6-1	*B. subtilis*	-
P7-2	*B. licheniformis*	++
P6-6	*B. licheniformis*	-
SS2-5	*B. subtilis*	-
P11-1	*B. pumilus*	-
P5-6	*B. subtilis*	++

Note: Zone of inhibition grading = - (size <0.9mm) insignificant inhibition, + (1-3mm) low inhibitory activity, ++ (size 3.1-4.9mm) moderate inhibitory activity, +++ (size >5.0mm) strong inhibitory activity.

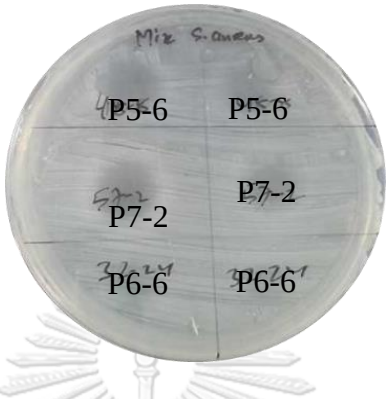


Figure 4.9 ZoI produced by cell free supernatant (CFS), correlating to the inhibitory action to gene over expression.

Table 4.7 and figure 4.9 shows the co-relation of gene expression with the inhibitory effect of cell free supernatant on cocktail *S. aureus.* CFS collected from the isolates were collected and filtered through 0.45µm filtrate. Then, 10µL 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 results showed only two of the isolates (P7-2 and P5-6) showed inhibitory action, respectively. Interestingly, this results co-relate to the gene expression results on agarose gel electrophoresis obtained from RNA product (Fig. 4.8A). As found in this study, at 5%NaCl, the gene expression assay was conducted after cultivating the bacterial cell for 24 hours when the RNA supposed to be disappeared, and the only two strains, *B. subtilis* (P5-6) and *B. licheniformis* (P7-2) could still exhibit the gene over expression. This demonstrated the strains might possess genetic property that can over express the gene expression.

According to the properties as discussed above, the isolates *B. subtilis* (P5-6) and *B. licheniformis* (P7-2), and condition using 5% NaCl and mixed *S. aureus* co-

culture were selected to further produce bacteriocin to develop as protective culture for application in food models.

4.3 Safety evaluation of selected isolates

Pariza and Foster (1983) stated the importance of food safety, including food enzymes, and in particular the importance to study on safety of candidate strains to be developed as food grade. The minimum safety assessment of the food microorganism should be determined for toxin production and hemolytic potential as recommended by Food and Agriculture Organization (FAO) US and European Food Safety Authority (EFSA, 2005). The safety evaluation of the selected isolates (*B. subtilis* and *B. licheniformis*) were tested for allergens (biogenic amines), including histamine, tyramine, putrescine, hemolysin production, pathogenicity, acute toxicity and cytotoxicity.

4.3.1 Evaluation of biogenic amines

Biogenic amines are functionally important low molecular weight nitrogenous bases and metabolic compounds in living organism and occasionally present in some food products causing considerable toxicological risks as potential human carcinogens when consumed in excess concentrations (Eom et al., 2015). The maximum allowable limit of biogenic amine (histamine) is 5.0mg/100g as suggested by US Food and Drug Administration (Food and Administration, 2011). Histamine, tyramine, putrescine and cadaverin are the main biogenic amines detected in fermented food products (Hernández-Orte et al., 2006). The consumption of accumulated biogenic amine in food is related to food poisoning (intoxication) with symptom including allergic reactions, nausea, vomiting, headaches, abdominal pain, and cardiac palpitation, increased or decreased blood pressure.

The presence of histamine, tyramine and putrescine in candidate strains was tested following multiplex PCR method, using primers mentioned by (Jeune et al., 1995, Coton et al., 2004, Landete et al., 2007). In the current study neither of the strains (*B. subtilis* or *B. licheniformis*) selected produced any of the mentioned biogeic amines. Although Han et al. (2007) reported that *B. subtilis*, *B. licheniformis*

and *B. amyloliquefaciens* were potential to have decarboxylase activites and could produce high biogenic amines from free amino acids available in fermentation system. However, the strains selected in this study, none of the biogenic amines (histamine, tyramine or putrecine) genes were found as shown in figure 4.10 A & B. Therefore these bacteria have no potential to genearte BA in fermentation system making it safe to be used in food systems in term of allergens production.

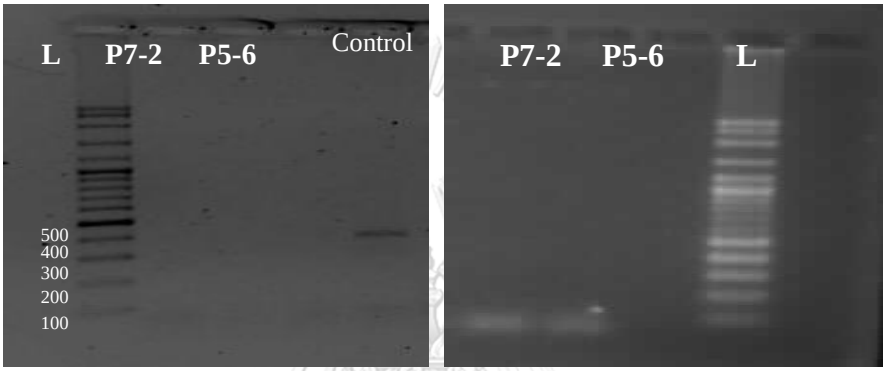


Fig. A Fig. B

Figure 4.10 1.5% agarose gel for biogenic amines production study

(A) Primer TD2/5 + TD-F/R + PUT2-F/R + HDC 3 / 4
(B) Primer set PUT1-F/R + TDC 1 / 2 + JV16HC/17HC

4.3.2 Hemolysin production evaluation

Hemolysin are the compounds that contributes to the pathogenicity of organism. The most extensively studied hemolysins produced by *Bacillus* strains are cereolysin (Hemolysin I) and hemolysin BL (HBL) beside other group of hemolysin enzymes produced (Mukry et al., 2010). Strains of *B. subtilis* and *B. cereus* are important as they cause food-spoilage and food-poisoning by producing hemolysin (subtilysin) enzymes apart from the production of biogenic amines (Bernheimer and Avigad, 1970). Thereby, screening of the hemolysin production of *Bacillus* spp. is important to avoid threat to food industry and public health. In this study, neither of the isolates (*B. subtilis* or *B. licheniformis*) produced any hemolysis, as shown in figure 4.11. The control used is an isolate of *Bacillus* that produce β-hemolysis,

where else the isolates P7-2 and P5-6 does not produce any zone of hemolysis or also called as λ-hemolysis (Savardi et al., 2018), considering it to be safe for use as food grade in term of non-hemolytic strains.

Figure 4.11 Blood agar to study hemolysis production.

An isolate of test organism was stabbed onto the blood agar plate and incubated at 37ºC overnight. The plate was inspected for any clear zone. As shown in the figure 4.11 the test organism P7-2 and P5-6 did not produce any clear zone but a control sample seems to have produced clear zone, due to the production of hemolysin. Therefore, based on the above results; negative for biogenic amines production and lack of toxin productions by the two isolates *B. subtilis* (P5-6) and *B. licheniformis* (P7-2), the choice was made to use *B. subtilis* for further study owing to following reasons. *Bacillus subtilis* is been accepted as food grade and used as starter for Natto (fermented soya bean product popular amongst Japanese population) for more than 100 years (Kubo et al., 2011, Steinkraus, 2004). It is also generally regarded as safe by US FDA and known to produce many active peptides (Harwood and Wipat, 1996, Apetroaie-Constantin et al., 2009). Moreover, as mentioned in table 4.6 above, isolate P5-6 (*B. subtilis*) exhibited larger zone of inhibition comparatively.

Therefore, hereafter the experiment was conducted using only single strain of *B. subtilis* (isolate P5-6) and to be selected for application into the food model system.

4.3.3 Acute toxicity and cytotoxicity testing

Along with the toxic production testing, the acute toxicity and cytotoxicity provides in-depth results of the microbes to be considered as food grade. *In vitro* study using cell lines, Caco2 and Vero cell cytotoxicity and *in vivo* exposure on experimental animals for acute toxicity. The results from BIOTEC and Department of Medical Science as shown in (Appendix D10-D12) non-cytotoxic and indicates the selected isolate of *B. subtilis* is accepted to be incorporated as food grade and safe for consumption.

Thus, based on the overall safety study and consumer acceptance as food grade organism, *B. subtilis* of isolate P5-6, isolated from salty fermented food (Plara) is further selected to be applied as food grade. Prior to application into food system, it is being made into ready to use protective culture by mixing with food grade matrix.

4.4 Application of isolates as protective culture for bio-control in foods

4.4.1 Determining MBC and MIC value

Prior to application of the isolates into the food system, it was prepared as ready to use protective culture. Minimum inhibitory concentration and minimum bactericidal concentration was determined for cell free supernatant following CLSI guideline. MIC is defined as the minimum amount of bacteriocin required to stop the proliferation of targeted bacteria (prevents the visible growth of bacteria) (Balouiri et al., 2016). MICs are used to evaluate the antimicrobial efficacy of various compounds by measuring the effect of decreasing concentrations of antibiotic over a defined period in terms of inhibition of microbial population growth. The Minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial

agent required to kill a bacterium over a specified period and conditions (Tong et al., 2014). The bacteriocin activity is expressed as Activity unit/Arbitrary units (AU) per milliliter (Pham et al., 2004). The MBC of the tested strain (*B. subtilis*) is found to be at 20AU/mL and MIC at 80AU/mL, as shown in table 4.8 and figure 4.12. The importance of estimating MBC and MIC will benefit to calculate the amount of protective culture to be added into the food system.

Table 4.8 MIC and MBC value of *B. subtilis*

Sam	Conc.	Double dilution AU/mL						
		10	20	40	80	160	320	540
Rep 1	0.485	0.059	0.066	0.072	0.237	0.375	0.492	0.484
Rep 2	0.360	0.056	0.070	0.081	0.295	0.320	0.362	0.372
Avg	0.422	0.057	0.068	0.076	0.266	0.345	0.427	0.428
			MBC		**MIC**			

Notes: Control contains only cocktail *S. aureus* (without bacteriocin), values are recorded by reading under 600nm wavelength, after overnight incubation ay 37°C.

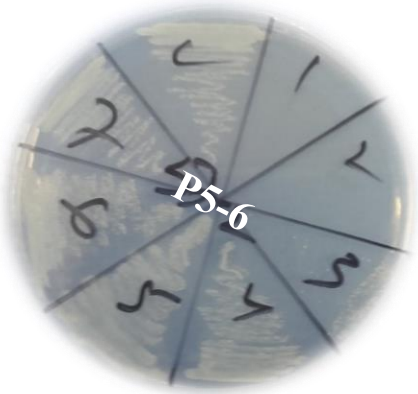


Figure 4.12 MBC of *B. subtilis*.

5-10µL of broth medium was taken from each well and cultured on NA+5%NaCl plate, incubated at 37°C overnight. Highest dilution without any visible growth is defined as MBC.

4.4.2 Protective culture application

Protective culture was prepared using freeze dried green mango pieces and skim milk as a powder matrices and protectants to be applied into two different food models. 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, similarly with the skim milk powder. The addition of culture was selected as protective culture (Leroy et al., 2003). The use of bacteriocinogenic *Bacillus* strains as protective culture 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 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). 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 skim milk powder protective culture and $2.7x10^6$CFU/g and a_w 0.6 in freeze dried mango pieces protective culture, respectively (shown in table 4.9). The protective culture were kept in the aluminium foil pack and stored at room temperature.

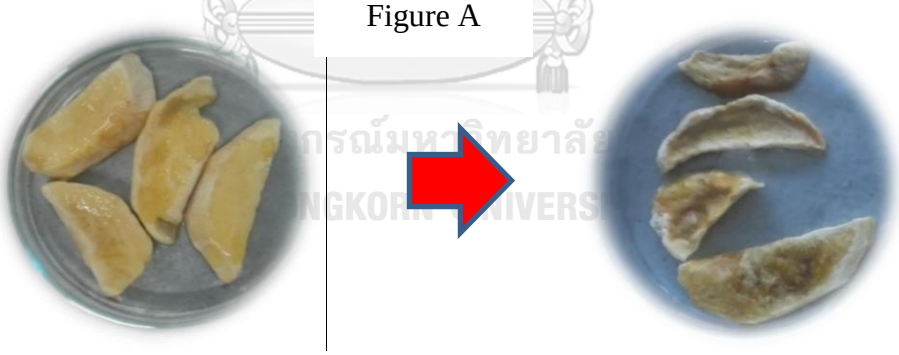
The idea of protective culture preparation was modified from Prakitchaiwattana et al., (2017) (trade secret). The MBC was confirmed again prior to application into the food and it was found to be at 20AU/mL for the both types of protective matrix (shown in table 4.9), similar to the MBC value mentioned in section 4.4.1.

Table 4.9 Properties of protective culture

Types of culture	Properties				
	Salt conc.	a_w	Cell survival (CFU/g)	MIC	MBC
Dried Skim milk powder	0.25% in 5mg	0.49±0.02	$1.8X10^6$	80AU/ml	20AU/ml
Dried Mango piece form	0.25% in 5mg	0.6±0.02	$2.7x10^6$	80AU/ml	20AU/ml

Values are mean ± standard deviation of duplicate sample determination

Figure A



Freeze dried mango pieces
+
Cell culture broth

Mango pieces protective culture

Figure B

Skim milk powder
+
Cell culture broth

Skim milk powder protective culture

Figure 4.13 Preparation of protective culture from two different food matrixes

Fig A. Freeze dried mango pieces to be used in bamboo shoot pickles.

Fig B. Skim milk powder to be used in cottage cheese.

4.4.3 Quality analysis of food samples

The food samples; bamboo shoot pickle manufactured locally at Tsirang**,** and cottage cheese manufactured at Bumthang, Bhutan were collected aseptically and transported to testing research laboratory for the study purpose. The cottage cheese (Datsi) is fresh consumed almost in a regular basis by the Bhutanese population. It is either added as seasoning in vegetable salads or cooked along with chili to make traditional Bhutanese dish ema-datsi. This type of cottage cheese has low shelf life (few days), therefore are usually stored in refrigerator by sprinkling salts to increase their shelf life, which otherwise is only few days. On the other hand, 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.

Table 4.10 Initial report of food samples analysis

Sample details	Ingredients	TPC CFU/ml	Yeast and Mold count CFU/ml	*S. aureus* count CFU/ml (Compact Disc)	pH[A]	NaCl Conc. mg/g %[A]	TTA %
Bamboo shoot pickle	Bamboo shoot, red chili, salt, and oil	$3.5x10^4$	<100	$1.6x10^3$	3.8±0.07	0.85±0.03	3.42
Green chili pickle	Green chili, oil and salt	$3.6x10^6$	<100	<100	3.6±0.02	1.2±0.23	3.57
Bumthang Local cheese	Curd milk	$1.7x10^6$	$1.2x10^4$	$2.5x10^5$	4.1±0.06	4.43±0.24	2.34
Bumthang Farm cheese	Curd milk	$8.3x10^7$	$7.2lx10^7$	$6.0x10^2$	4.2±0.02	3.2±0.25	2.6
Gelephu local cheese	Curd milk	$9.2x10^7$	$4.4X10^6$	$3.2x10^3$	4.1±0.02	2.2±0.21	2.08

Note: [A] Values are mean ± standard deviation of duplicate sample determination. The highlighted samples are the samples that is been used in the current work for addition of protective culture.

Microbiological analysis, initial total plate count, and yeast and mold count was performed, along with some general properties, including pH, total tritatable acidity (TTA) and salt content. Safety evaluation was done for *S. aureus* contamination. Table 4.10 shows the complete report for food sample analysis.

Both the food samples (cheese and pickle) are acidic foods. Generally, in foods having low pH, only few microorganism, including *S. aureus* can survive the low pH, which is other pathogenic bacteria's supposed to be inhibited (Radford and Board, 1993). However, in the sample tested, the presence of *S. aureus* in food samples were still found up to log 5 CFU/g in the cottage cheese. The fate of the home made products usually have the high contamination with *S. aureus* due to low hygienic practice, and also because this cheese ball (Datsi) is made by using bare hands, thereby transferring microbes from hands into the cheese. Studies done by Singh and Prakash (2008) and De Luca et al. (1997) mentioned that *S. aureus*

contamination is highly found in milk products and cheese is a common source for food poisoning due to staphylococcal intoxication (Wang et al., 2013, Zeleny et al., 2015). Almost everyone can be susceptible and get infected with staphylococcal food poisoning. The severity depends on the amount of toxins ingested or also protective immunity of the individual (Argudín et al., 2010). The person with low immunity and young adults may develop serious infection requiring hospital administration. Therefore, to prevent the contamination of food due to *S. aureus* is important. These selected food samples, despite being acidic 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.

The low pH was observed is bamboo shoot pickle and this is due to lactic acid fermentation as members of LAB and some molds are present in bamboo shoot fermentation (Thakur et al., 2016). The amount of salt present in food samples were found to be around 5% in cheese and 1% in pickle, but this does not remain constant in all the products manufactured, as the villagers does not follow a specific guideline to control the amount and type of salt to be added.

4.5 Application of protective culture as bio-control in food

In this part, the protective culture of *B. subtilis*, with negative toxicity results and having GRAS property with history of safe use (Teo and Tan, 2005) made as ready to use, so called protective culture was added to the food samples. One-gram protective culture added to 9g of samples (1:10 ratio). The protective culture was added 10 times the MBC value to withstand and adapt the food matrix.

4.5.1 Cheese

After the addition of protective culture, the cheese samples were formed into ball shape and kept at refrigerated temperature (4-6°C) until further analysis. The cheese samples were tested every 4 days for physical properties, along with TPC, *S. aureus* count and pH. The samples were repeated with control. It was found that there were no detectable physical changes observed between the controls and samples

when stored at 4-6°C refrigerated temperature until day 12, but at the end of the 16th day, the controls started slime formation around the external surfaces and developed strong off-odor. Whereas, the samples with protective culture still appeared fresh. Therefore, the addition of protective culture helps to maintain the freshness of cottage cheese for longer duration.

The microbiological analysis found that the initial load of *S. aureus* in cottage cheese was at log 5 and remained constant throughout the storage period at log 4 in controls. The pH was recorded to be 4.14 at the initial analysis, as shown in the table 4.10. The final pH is recorded at 4.2 in control and 4.4 in sample at the end day16, not significantly different at $p<0.05$. *Staphylococcus aureus* total viable count was found to be at log 5 initially from the fresh sample. In control sample, the count decreased to log 4 on day 4, then the *S. aureus* count remained almost at constant rate log 3 to over log 4 during 8, 12 and 16th day. The *S. aureus* count in samples supplemented with protective culture decreased to log 3 at day 4. Thereafter the *S. aureus* count decreased to below log 2 at day 12 and day 16, respectively. This indicated the successful production and effectiveness of bacteriocin produced by the *Bacillus* species (*B. subtilis*) in cottage cheese. Though the *S. aureus* count in final sample was still within the detectable range but it's thought that increasing the amount of protective culture into the cheese could probably bring the count lower below the detectable level.

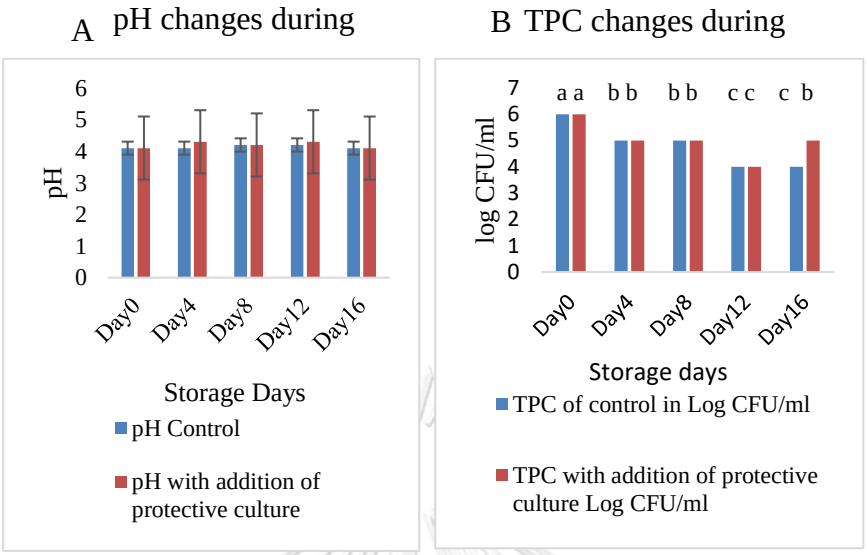


Figure 4.14 Effect of addition of protective culture in cottage cheese

Fig A. Change in pH over the storage days. The change in the pH from the initial to the storage until 16th day was insignificant at p<0.05

Fig B. Change in TPC over the storage days. The change in total plate count from the initial sample and the final sample at the end of 16th day was insignificant at p<0.05.

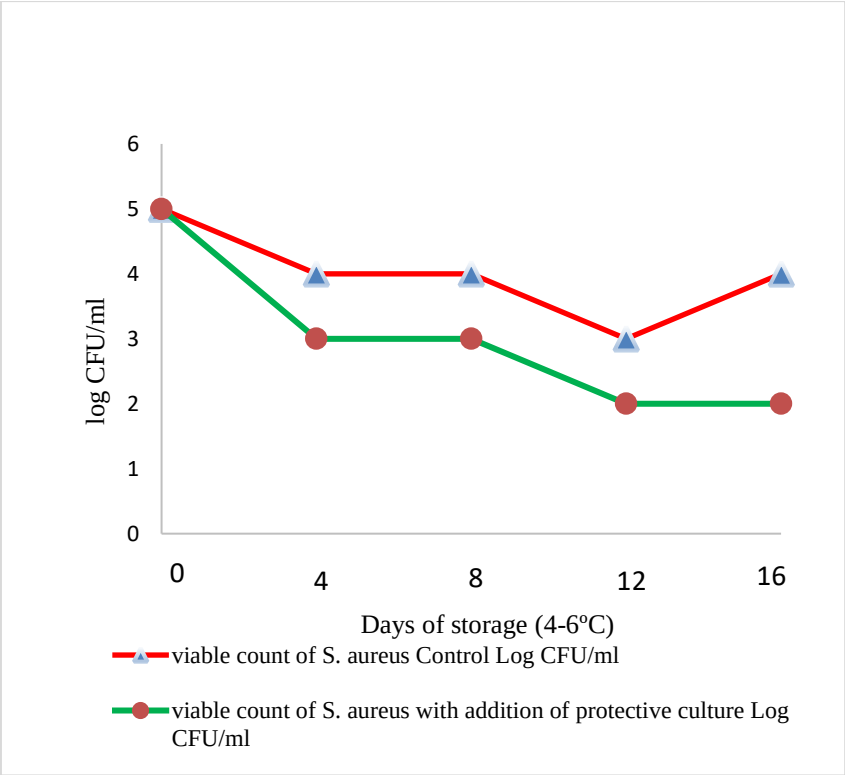


Figure 4.15 *Staphylococcus aureus* count of cheese sample, stored at 4-6°C for 16 days

The initial *S. aureus* population in control and sample was recorded at log 5. The count decreased lo log 4 in control at day 4 and the count in sample decreased to log 3, a decrease by 2 log due to the activity of antimicrobial compounds produced by protective culture. Subsequently the count in sample reduced to log 2 at the end of day 16, were else the count in the control was recorded at log 4.

4.5.2 Pickle

Similarly the green mango pieces proetective culture were 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 physical examination 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* protective culture did not produce too much of hydrogen sulphite or otherwise will have changed the color

to dark brown. The pH value between the samples and the control recored at 4th weeks was 3.9 in control and 3.8 in samples, not significant different at $p<0.05$. The TPC ranged from log 3 and log 4. Initially the amount of *S. aureus* in pickle was detected at log 3 and the count reduced to <100 CFU/mL after addition of protective culture after week 2, while the pathogen in control still remained at 3 log CFU/mL throughout the storaged days. Again, this indicateed the achivement in this protective stater culture of *B. subtilis* to control the *S. aureus* in the bamboo shoot pickle.

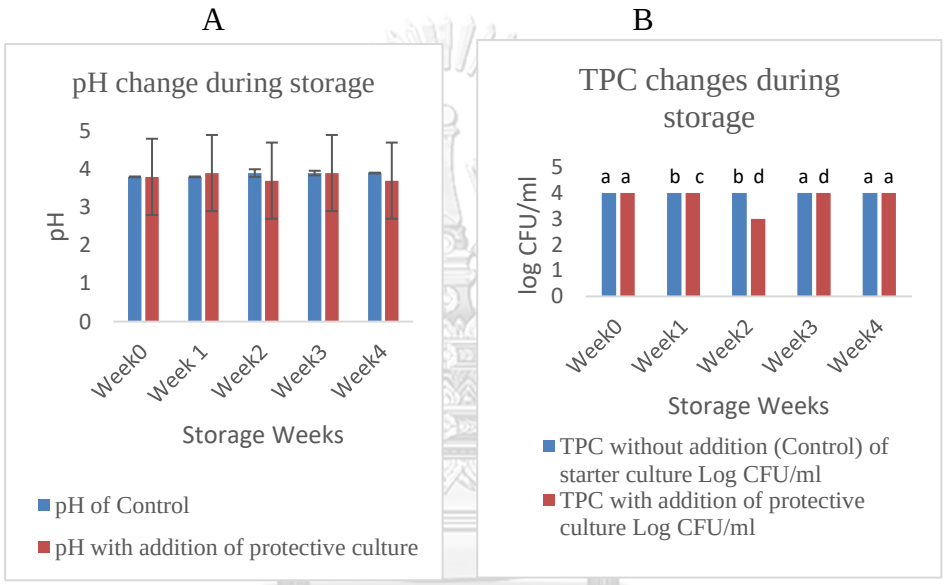


Figure 4.16 Effect of addition of protective culture in bamboo shoot pickle

Fig A. Change in pH over the storage weeks. The change in the pH from the initial to the storage until 4 weeks was insignificant at $p<0.05$

Fig B. Change in TPC over the storage weeks. The change in the total plate count from the initial to the storage until week 4 was insignificant at $p<0.05$

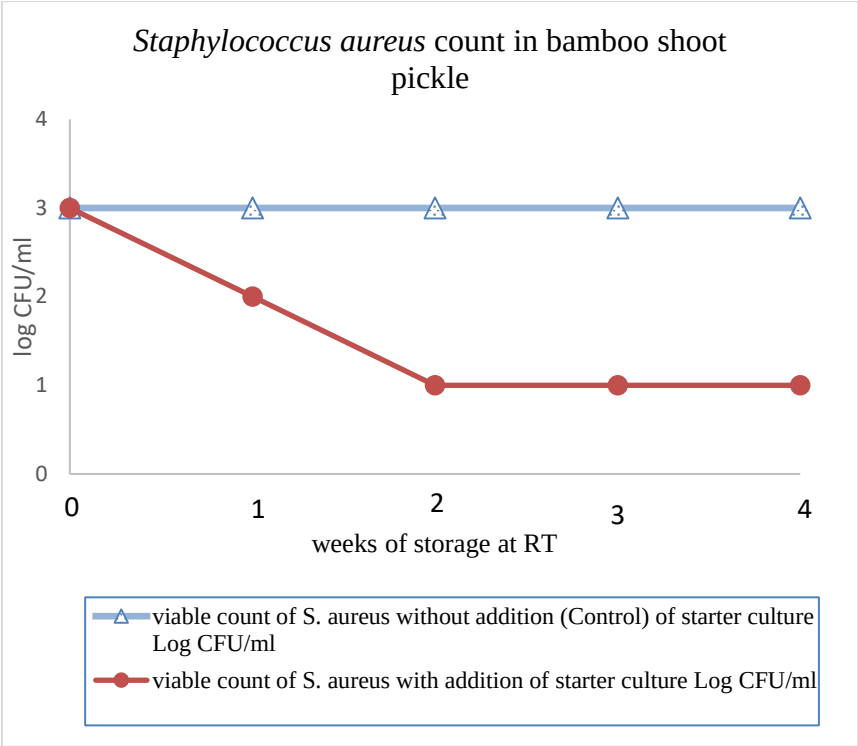


Figure 4.17 *Staphylococcus aureus* count of bamboo shoot pickle sample, stored at RT for 4 week**s.**

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.

Finally, it can be concluded that the addition of *B. subtilis* in the form of protective culture did reduce contaminating *S. aureus* in food system. This could be related to the release of bacteriocin by *B. subtilis*, thereby indicating the success of using *B. subtilis* as a protective bio-preservative in food system.

CHAPTER 5

CONCLUSION

This research was a supplementary study on the projects 'development of specific functional starter culture for Plara and soya sauce', with the aim to isolate halophile bacteria with active inhibitory characteristic onto contaminating pathogens, especially, targeting *S. aureus* which is common halotolerant pathogen. Initial study was done following both culture dependent and cultural independent technique, to study bacteria present in the Plara and soya sauce sample ecosystem. The results from the two methods showed completely different microbial isolates. Species of *Bacillus* were the main isolates found by convention cultural plating method on both the samples. On the other hands, *Staphylococcus gallinarium* and Uncultured *Halanaerobium* spp. were the main isolated detected by culture independent Rev-T-DGGE method, in soya sauce and Plara sample, respectively.

From 124 isolates, six different species of genus *Bacillus*, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus, B. atrophaeus* and *B. altitudinis* were the main isolates observed in both salty fermented systems. These *Bacillus* isolates exhibited inhibitory activity on three different strains of *S. aureus* used as target organism but with different pattern, significantly different at $p<0.5$. The *Bacillus* isolates with inhibitory activity were evaluated for gene encoding subtilin (*spaS*) and subtilosin (*sboA*), common bacteriocin produced by *Bacillus* genus. There were 37 isolates exhibiting inhibitory activity but only 9 isolates that contained subtilin or subtilosin or both the genes. Furthermore, an isolate of *B. subtilis* and *B. lichenoformis* presented with over gene expression in 5% NaCl concentration and cocktail *S. aureus* co-culture in nutrient broth.

Prior to application of these *Bacillus* strains into food system. It was evaluated for toxicity testing. Common biogenic amines histamine, tyramine and putrecine were found to be negative conducted using multiplex PCR. Ready to use protective was prepared with *B. subtilis* (isolate P5-6) culture, using skim milk powder and freeze dried green mango pieces as matrix by two step drying temperature (55°C and 50°C), and to be applied as protective culture into two different food models; cottage

cheese and bamboo shoot pickle. Firstly, application in cottage cheese presented the reduction of *S. aureus* from 5 log CFU/g to lower 2 log CFU/g, in addition with, significantly helped prolong the freshness of the cheese. Secondly, application in bamboo shoot pickle reduction of *S. aureus* from 3 log CFU/g to lower 1 log CFU/g without impact on the other properties of the pickle.

Moreover, as mention earlier, different isolates of *Bacillus* bacteriocin producer strains/species were located in different cluster of phylogenetic tree. This finding is relatively novel that need to further study whether the genetic evolution of the *Bacillus* group associated to the defensive mechanisms to survive in the natural habitats. This reflects the potential of this bacterial group to be developed as a bio-control agent for example in food manufacturing.

Therefore, these results suggested that the halophile *B. subtilis* isolated from salty food systems could probably produce subtilosin (confirmed by the gene expression along with inhibitory action having activity against *S. aureus)*, and to be considered safe to apply as bio-control agent in term of protective culture. Subtilosin is lantibiotics bacteriocin and acts as bactericidal agent by action on the inhibition of cell wall synthesis or formation of membrane pores. Moreover, the toxicity testing revealed that the strain didn't produce any allergen or toxic substances, adding a value to its GRAS property.

Recommendation: the detail study about the produced bacteriocin characteristics, such as structure, its stability to different temperature, pH and other factors, along with the more toxicology study by sub-cloning onto mouse and certification to use in food system would be considered for further development for industrial application in future studies.

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APPENDIX

APPENDIX A.
Molecular procedures

APPENDIX A1
Total RNA extraction

1. Bacterial cells (7.5 10^{7-8}CFU/ml)
2. Centrifuge 12,000rpm for 2 minutes
3. Suspend in 400µl isolation buffer (10mM EDTA, 50mM Tris-HCL, 5% SDS, pH 6.0)
4. Incubate at 65°C, 5 minutes in water bath
5. Cool in ice
6. Add 300µl Lysis buffer + β-mercaptoethanol or DTT (20µl of 14.3 β-mercaptoethanol or 2 M DTT to each 1ml of lysis buffer)
7. Add 600µl proteinase K (10µl proteinase K + 950µl TE buffer (10mM Tris HCL, pH 8 + 1mM EDTA))
8. Vortex and incubate in 15-25°C for 10 minutes
9. Transfer to new tube
10. Add 450µl 96-100% ethanol
11. Transfer 700µl lysate to column inserted in collection tube
12. Centrifuge 1 minute at 12,000rpm
13. Discard the flow through
14. Repeat with the remaining lysate (step 12 & 13)
15. Add 700µl wash buffer 1 (supplemented with 10ml 96-100% ethanol to each 40ml wash buffer)
16. Centrifuge 1 minute at 12,000rpm
17. Discard the flow through
18. Add 100µl of DNase I (diluted 1:3 with PCR water), allow to stand at RT for 10 minutes
19. Add 600µl of wash buffer 2 (supplemented with 39ml 96-100% ethanol to each 23ml of wash buffer)
20. Centrifuge 1 minute at 12,000rpm
21. Discard the flow through
22. Add 250ml of wash buffer 2
23. Centrifuge 2 minutes at 12,000rpm
24. Discard the flow through
25. Place the column in new collection tube
26. Add 50µl of nuclease free water, stand for 3 minutes
27. Centrifuge 1 minute at 12,000rpm
28. Store RNA at -20 to -80°C or convert to Cdna

APPENDIX A2
Conventional RNA Extraction protocol (Li et al., 2009)

1. Bacterial cells (10^{7-8} CFU/ml)
2. Centrifuge 12,000 rpm, 2 minutes
3. Suspend in 400µl isolation buffer (10mM EDTA, 50mM Tris-HCL, 5% SDS, pH6)
4. Incubate 65°C 5 minutes
5. Cool in ice
6. Add 200 µl 0.3M KCl, pH 6
7. Mix, centrifuge 12,000, 10 minutes at 4°C
8. Transfer supernatant to new tube
9. Add equal volume of phenol/chloroform/isoamylalcohol (25:4:1)
10. Mix, centrifuge 12,000 5 minutes at 4°C
11. Transfer supernatant to new tube
12. Add 0.1 volume sodium acetate, pH5.2 + 2.5 volume ethanol
13. Incubate -20°C, 10 minutes
14. Mix, centrifuge 13,000 10 minutes at 4°C
15. Wash pellet with 70% ethanol
16. Mix, centrifuge 13,000 5 minutes at 4°C
17. Air-dried
18. Re-suspend in 50µl DEPC water
19. Store -70°C until use

APPENDIX A3
Total DNA extraction (Dashti et al., 2009)

1. Transfer 1000µl of cell suspension
2. Centrifuge at 10,000rpm for 15 minutes, transfer the pellets into sterile micro vials
3. Mix with 500µl lysis buffer (50mM Tris, 10mM EDTA, 2%SDS, pH 8.0)
4. Incubate at 60°C for 1 hour
5. Add 500µl of phenol-chloroform (1:1), mixed
6. Centrifuge 10,000 rpm for 15 minutes
7. Transfer the supernatant, add equal volume of chloroform
8. Centrifuge at 10,000rpm for 15 minutes
9. Transfer supernatant to clean tube
10. Add equal volume of isopropanol, mix
11. Stand in an ice-water bath for 30 minutes
12. Centrifuge at 10,000rpm for 20 minutes
13. Wash pellet with ice-cold 70% ethanol
14. Centrifuge at 10,000rpm for 15 minutes
15. Air dry
16. Dissolve in 50µl TE (Tris-HCL 10mM pH 7.6, edta 1 mM pH 8.0)
17. DNA sample was stored at -18°C.

APPENDIX A4

PCR reaction master mix preparation

Reagent	Concentration		Volume		Thermal cycling program
	Stock	Working	Work 50ul	Total -- Reac	
Vi buffer	10X	1X	5		Lid:80°C Volume:50ul 1. 94°C, 2:00 Min 2. 94°C, 0:30 Sec 3. 55°C,0:30 Sec 4. 72°C,0:30 Sec 5. Go To STEP 2, 35X 6. 72°C, 7:00 Min 7. 4°C, ∞
$MgCl_2$	50mM	1.5mM	1.5		
dNTPs	10uM	0.1mM	0.5		
Primer	F-10uM	0.1mM	0.5		
	R-10uM	0.1mM	0.5		
Tag DNA	5U/ul	2U	0.4		
H_2O	-	-			
Template	-	10-50ng/ul	0.4		

Reagent (Reverse Transcriptase)	Concentration	Volume		Thermal cycling program
		Work 10ul	Total Reaction	
dNTPs	10U	1		Lid: 80°C Volume: 10ul 1. 65°C, 5 Min 2. 4°C, ∞
Oligo d	40um	1		
Hexamer		1		
RNA free H_2O	-	5		
Template	10-50ng/ul	2		
10X buffer		2		Lid: 80°C Volume 20ul 1. 42°C, 60 Min 2. 4°C, ∞
Reverse Transcriptase		0.5		
RNA free H_2O	-	7.5		

Reagents	8% Denaturation gel (DGGE)	
	20	40
40% Acrylamide/Bis	3ml	3ml
50XTAE	0.3ml	0.3ml
Formamide	1.2ml	2.4ml
Urea (g)	1.26g	2.52g
Glycerol	0.3ml	0.3ml
RNA free H_2O	10.2ml	9ml
TEMED	0.0154ml	0.0154ml
10%APS	0.154ml	0.154ml

APPENDIX B
Cultural Media preparation

B1. Nutrient agar: dissolve 28.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

B2. MRS agar: dissolve 67.15 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

B3. Dissolve 24.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates. Acidify the medium with sterile 10% tartaric acid. The amount of acid required for 100ml of sterile cooled medium is approximately 1ml. Do not heat the medium after addition of acid.

B4. 0.5%Peptone water: dissolve 5 grams of peptone powder in 1000ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15lbs pressure (121°C) for 15 minutes.

APPENDIX C
Determination of microbiological property

C1. Yeast and Mold count (A.O.A.C., 1995)

1. Take 1ml of sample into 9ml 0.1% peptone water
2. Make serial dilution of the samples
3. Spread plate 0.1ml of the sample diluted onto the PDA plate (supplemented with 10% tartaric acid)
4. Incubate at 30°C for 2-3days
5. Colony count

C2. Bacterial count (A.O.A.C., 1995)

1. Take 1ml of sample into 9ml 0.1% peptone water
2. Make serial dilution of the samples
3. Spread plate 0.1ml of the sample diluted onto the Nutrient agar (supplemented with 5% NaCl)
4. Incubate at 30°C for 2-3days
5. Colony count

APPENDIX D

APPENDIX D1

Standard curve of bacterial population

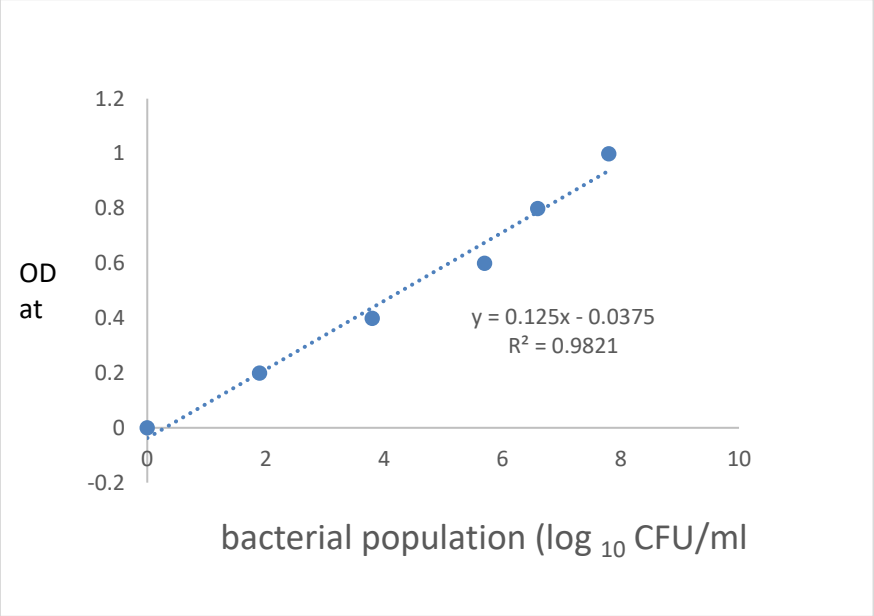


Figure D1, the relationship between bacterial population (log 10 CFU/ml) and absorbance values (λ_{max} 600nm)

Calculation

The linear equation: y= 0.125x-0.0375

Where y=absorbance at 600nm

X=bacterial population

APPENDIX D2

Standard curve of salt concentration

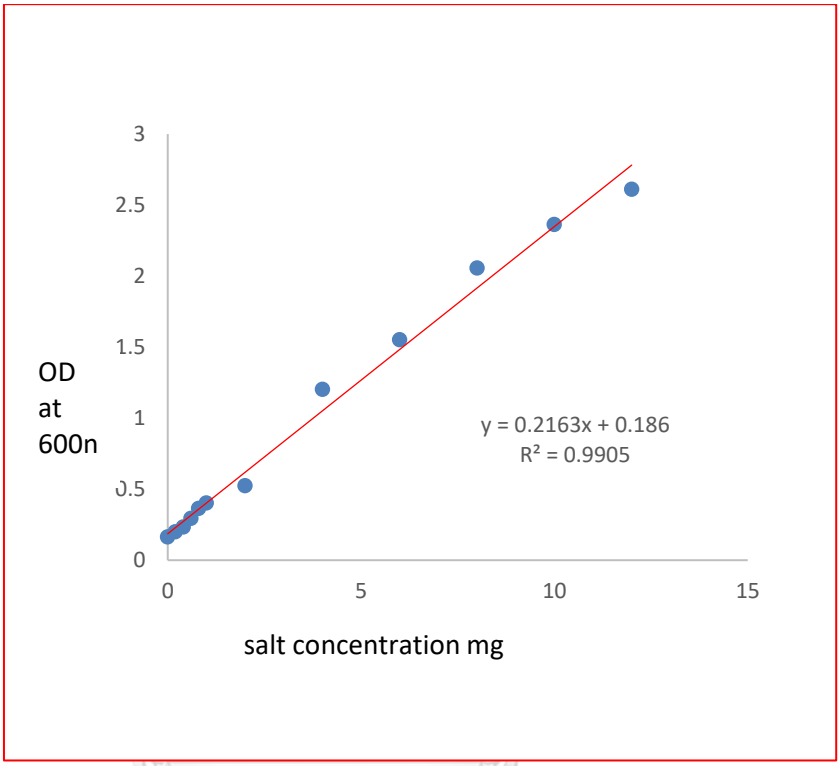


Figure D2, the relationship between salt concentration and absorbance values (λ_{max} 600nm)

Calculation

The linear equation: y= 0.2163x+0.186

Where y=absorbance at 600nm

X=salt concentration

APPENDIX D3

Table Zone of inhibition of *Bacillus* species on different *S. aureus* strains

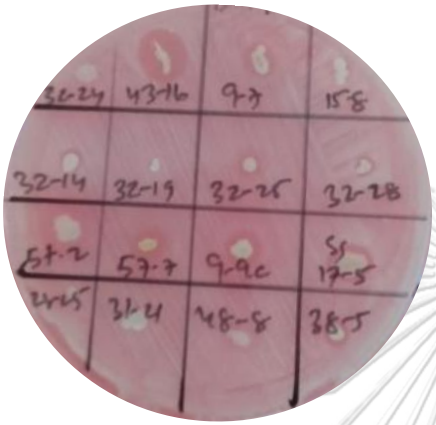
Code	Isolate spp.	ATCC	DMSc	Food isolate	Mix
P6-1	*B. subtilis*	0.60±0.10^{fghij}	0.73±0.06^{gh}	0.70±0.10^{fg}	0.60±0.20^{fgh}
SS2-5	*B. subtilis*	0.53±0.12^{defgh}	0.53±0.06^{cdefg}	0.63±0.06^{efg}	0.43±0.06^{def}
P3-11	*B. subtilis*	0.33±0.06^{bcdef}	0.53±0.12^{cdefg}	0.47±0.12^{cdef}	0.53±0.12^{efgh}
P16-1	*B. subtilis*	0.27±0.23^{bcd}	0.40±0.00^{bcde}	0.00^{a}	0.50±0.10^{defgh}
P10-3	*B. subtilis*	0.40±0.00^{bcdefg}	0.37±0.06^{bcd}	0.47±0.12^{cdef}	0.30±0.00^{bcd}
P6-2	*B. subtilis*	0.33±0.06^{bcdef}	0.33±0.06^{bcd}	0.00^{a}	0.37±0.06^{bcde}
P6-12	*B. subtilis*	0.00^{a}	0.50±0.10^{bcdefg}	0.00^{a}	0.00^{a}
P4-9	*B. subtilis*	0.83±0.15^{j}	0.87±0.12^{h}	0.73±0.12^{fg}	0.37±0.06^{bcde}
P8-1	*B. subtilis*	0.53±0.12^{defgh}	0.47±0.06^{bcdefg}	0.33±0.11^{bcd}	0.37±0.06^{bcde}
P8-2	*B. subtilis*	0.37±0.06^{bcdefg}	0.43±0.15^{bcdef}	0.53±0.12^{cdef}	0.23±0.11^{bc}
P1-3	*B. subtilis*	0.37±0.15^{bcdefg}	0.47±0.31^{bcdefg}	0.00^{a}	0.30±0.00^{bcd}
P6-9	*B. subtilis*	0.80±0.20^{ij}	0.87±0.12^{h}	0.83±0.12^{g}	0.40±0.00^{cde}
P5-6	*B. subtilis*	0.73±0.12^{hij}	0.70±0.10^{fgh}	1.07±0.12^{h}	0.67±0.06
P1-4	*B. subtilis*	0.47±0.12^{cdefgh}	0.53±0.12^{cdefg}	0.00^{a}	0.00^{a}
P15-1	*B. subtilis*	0.60+0.20^{fghij}	0.27±0.03^{bc}	0.33±0.06^{bcd}	0.00^{a}
P6-5	*B. amyloliquefaciens*	0.57±0.06^{bcdef}	0.37±0.06^{bc}	0.40±0.00^{bc}	0.43±0.06^{efgh}
P6-11	*B. amyloliquefaciens*	0.20±0.10^{abc}	0.00^{a}	0.30±0.00^{bc}	0.43±0.06^{def}
P9-2	*B. amyloliquefaciens*	0.27±0.06^{bcd}	0.43±0.15^{bcdef}	0.00^{a}	0.00^{a}
P9-1	*B. amyloliquefaciens*	0.20±0.10^{abc}	0.00^{a}	0.20±0.10^{ab}	0.43±0.06^{def}
P15-2	*B. amyloliquefaciens*	0.57±0.06^{efghi}	0.47±0.15^{bcdefg}	0.47±0.40^{cdef}	0.53±0.06^{efgh}
P17-1	*B. amyloliquefaciens*	0.57±0.06^{efghi}	0.37±0.06^{bcd}	0.40±0.00^{bcde}	0.43±0.06^{def}
P13-1	*B. pumilus*	0.47±0.12^{cdefgh}	0.40±0.00^{bcde}	0.00^{a}	0.33±0.06^{bcde}
P14-2	*B. pumilus*	0.33±0.06^{bcdef}	0.67±0.12^{efgh}	0.60±0.26^{defg}	0.67±0.12^{h}
P12-3	*B. pumilus*	0.43±0.15^{bcdefg}	0.70±0.10^{fgh}	0.40±0.00^{bcde}	0.47±0.06^{defg}
P12-4	*B. pumilus*	0.30±0.10^{bcde}	0.23±0.10^{ab}	0.00^{a}	0.37±0.10^{bcde}
P1-6	*B. pumilus*	0.43±0.15^{bcdefg}	0.47±0.12^{bcdefg}	0.50±0.26^{cdef}	0.37±0.06^{bcde}
P7-5	*B. pumilus*	0.37±0.06^{bcdefg}	0.33±0.06^{bcd}	0.00^{a}	0.33±0.06^{bcde}
P11-1	*B. pumilus*	0.27±0.10^{bcd}	0.47±0.12^{bcdefg}	0.00^{a}	0.33±0.06^{bcde}
P7-2	*B. licheniformis*	0.47±0.12^{cdefgh}	0.37±0.02^{bcd}	0.47±0.12^{cdef}	0.20±0.10^{b}
P10-7	*B. licheniformis*	0.27±0.12^{bcd}	0.43±0.15^{bcdef}	0.40±0.17^{bcde}	0.33±0.06^{bcde}
P6-6	*B. licheniformis*	0.17±0.02^{ab}	0.23±0.11^{ab}	0.20±0.10^{ab}	0.00^{a}
P7-1	*B. licheniformis*	0.33±0.06^{bcdef}	0.00^{a}	0.00^{a}	0.00^{a}
P12-1	*B. atrophaeus*	0.63±0.06^{ghij}	0.40±0.00^{bcde}	0.57±0.15^{cdef}	0.53±0.12^{h}
P4-2	*B. atrophaeus*	0.33±0.06^{bcdef}	0.00^{a}	0.47±0.21^{cdef}	0.30±0.00^{bcd}
P14-1	*B. altitudies*	0.60±0.20^{fghij}	0.57±0.21^{defg}	0.57±0.06^{cdef}	0.33±0.06^{bcde}
P6-8	*Lelliottia*	0.60±0.20^{fghij}	0.47±0.12^{cdefg}	0.67±0.12^{efg}	0.63±0.06^{gh}
P3-1	*S. epidermidis*	0.47±0.12^{cdefgh}	0.67±0.12^{efgh}	0.57±0.06^{cdef}	0.47±0.12^{defg}

Different lower-case superscripts within a column indicate significant difference (p<0.05)

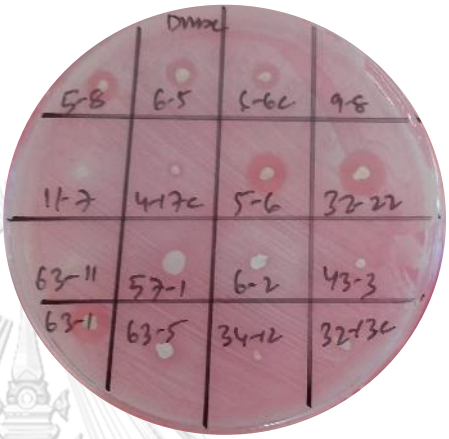
Values are mean ± standard deviation of triplicate sample determination

APPENDIX D4

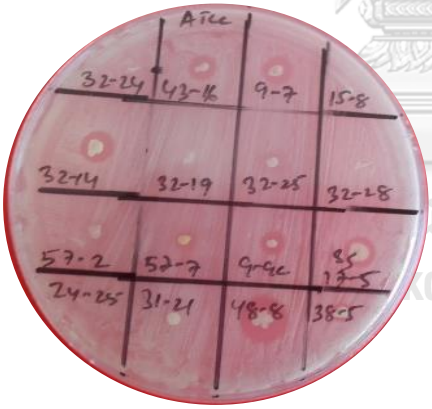
ZoI of *Bacillus* isolates to different *S. aureus* on nutrient agar



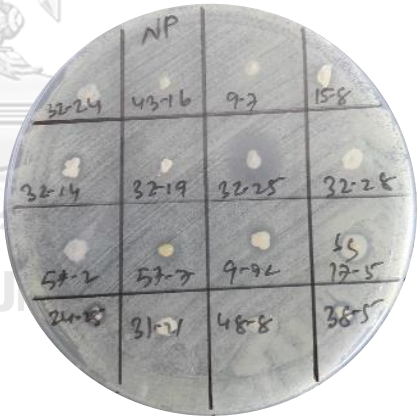
A. (Mix/cocktail *S. aureus*)



B. (DMSc 6538 *S. aureus*)



C. ATCC 25922 *S. aureus*



D. Food grade *S. aureus* (FT30-7)

APPENDIX D5

Table Sequencing Results of DGGE Bands

DGGE Band (ID)	Sequences	Isolate ID	% Homo	Accession no.
A	CCGCTTTCACTGGGCGCAGC CTGATGGAGCACGCCGCGTG AGTGAAGACGGTCTTCGGAT TGTAAAGCTCTGTCCTTAGG GAAGAACCGTGGGTATAGC AAATGATACCCATCTGACGG TACCTTTGGAGGAAGCACTG GCTAACTACGTGCCAGCAGC CGCGGTAATAACT	Uncultured *Halanaerobium* spp.	98	KU961746 .1
B	CCGCTGCACTGGTCGCAGTC TGATGGAGCAACGCCGCGT GTATGAAGAAGGTCTTCGGA TCGTAAAATACTGTTGTCAG AGAAGAACACGTGATAGAG TAACTGTTATGGCGCTGACG GTATCTGACCAGCAAGTCAC GGCTAACTACGTGCCAGCAG CCGCGGTAATACGT	*Lactobacillus acidpiscis*	98	KX139191 .1
C	CCGCTTTCGCATGGGCGAAG CCTGACGGAGCACGCCGCGT GAGTGATGAAGGGTTTCGGC TCGTAAAACTCTGTTATTAG GGAAGAACATATGTGTAAG TAACTGTGCACATCTTGACG GTACCTAATCAGAAAGCCAC GGCTAACTACGTGCCAGCAG CCGCGGTAAT	*Staphylococcus gallinarum*	98	MF399027 .1
D	GAGCTTTACGATCCGAAGAC CTTCTTCACACACGCGGCGT GGCTGCATCAGGACTTGCGC TCCATTGTGGAAGATTCCCT ACTGCTGCCTCCCGTAGGCG AGCCGAACCCTTCGTCCTCA CGCGGCGTTGCTCGACCAGC CTTGCGTCCATTGTGGAAGA TTCCCTACCGCTGCCTCCCG TAGGCGCGCCGTGCCCCAGC CTAGCCCGCCG	*Lactobacillus delbrueckii*	95	MF446929 .1

APPENDIX D6

Nucleotide sequencing analysis

Code	Nucleotide sequence	Spp. ID	%Homo	Accession no.
P13-1	GCGGTATCGCATGGAGAAGTCTGACG GAGCACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCGAGAGTAACTGC TCGCACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. pumilus*	99	HM216571.1
P14-2	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAAT	*B. pumilus*	98	HM055957.1
P12-3	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATTA	*B. pumilus*	99	HM055957.1
P12-4	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAAT	*B. pumilus*	99	HM055957.1
P1-3	GGACATCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGA	*B. subtilis*	100	GU434356.1
P1-4	GGGCATCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCCGTTCAAATAGG GCGGCACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATA	*B. subtilis*	100	GU434362.1
P1-6	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATGA	*B. pumilus*	99	HM055957.1
P15-1	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. subtilis*	99	HQ268531.1
P11-1	CCACATCGCATGGACGAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT	*B. pumilus*	99	HM055978.1

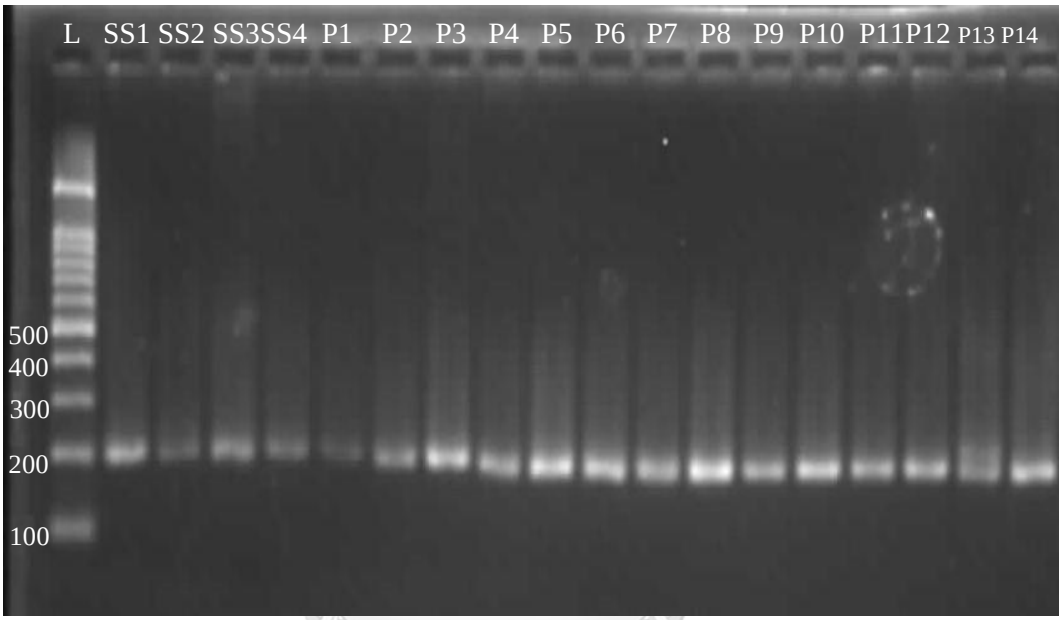
	TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAAT			
SS2-5	GCGGTTTCGCATGGAGAAGTCTGACG GAGCACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCCGTTCAAATAGG GCGGCACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAAT	*B. subtilis*	99	HQ333016.1
P3-11	GGCTTCGCATGGAGAAGTCTGACGGA GCACGCCGCGTGAGTGATGAAGGTTT TCGGATCGTAAAGCTCTGTTGTTAGGG AAGAACAAGTACCGTTCGAATAGGGC GGTACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATT	*B. subtilis*	99	HQ268531.1
P16-1	GGGCTTCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCCGTTCAAATAGG GCGGCACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATAGA	*B. subtilis*	99	AY659857.1
P10-3	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGAAGAAGGTT TTCGGATCGTAAAACTCTGTTGTTAGG GAAGAACAAGTGCCGTTCGAATAGGG CGGCGCCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATA	*B. subtilis*	99	HQ268531.1
P6-1	GGGCTTCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAACTCTGTTGTTAG GGAAGAACAAGTACCGTTCGAATAGG GCGGTACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATA	*B. subtilis*	99	GU434356.1
P6-2	GGGCTTCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCCGTTCAAATAGG GCGGCACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATA	*B. subtilis*	99	AY659857.1
P6-5	GGGGTTTTCGCATGGAGAAGTCTGAC GGAGCACGCCGCGTGAGTGATGAAGG TTTTCGGATCGTAAAGCTCTGTTGTTA GGGAAGAACAAGTGCCGTTCAAATAG GGCGGCACCTTGACGGTACCTAACCA GAAAGCCACGGCTAACTACGTGCCAG CAGCCGCGGTAATT	*B. amyloliquefaciens*	100	KF611911.1
P6-6	GGACTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAACTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. licheniformis*	98	KU870772.1
P6-8	GGCAGCATGGGCGCAGCCTGATGCAG CCATGCCGCGTGTATGAAGAAGGCCT TCGGGTTGTAAAGTACTTTCAGCGAG GAGGAAGGCATTGCGGTTAATAACCG	*Lelliottia spp*	99	KJ810589.1

	CAGTGATTGACGTTACTCGCAGAAGA AGCACCGGCTAACTCCGTGCCAGCAG CCGCGGTAATT			
P6-12	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCGAGAGTAACTGCT CGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATTGA	*B. subtilis*	98	KC595863.1
P6-9	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. subtilis*	99	HQ268531.1
P6-11	GGGGCATTTCGCATGGAGAAGTCTGA CGGAGCACGCCGCGTGAGTGATGAAG GTTTTCGGATCGTAAAGCTCTGTTGTT AGGGAAGAACAAGTGCCGTTCAAATA GGGCGGCACCTTGACGGTACCTAACC AGAAAGCCACGGCTAACTACGTGCCA GCAGCCGCGGTAATTA	*B. amyliliquefaciens*	99	KF611911.1
P9-2	GGGCATCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAACTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATTA	*B. amyloliquefaciens*	98	KF611911.1
P9-1	GGCTTCGCATGGAGAAGTCTGACGGA GCACGCCGCGTGAGTGATGAAGGTTT TCGGATCGTAAAGCTCTGTTGTTAGGG AAGAACAAGTGCCGTTCAAATAGGGC GGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATT	*B. amyloliquefaciens*	99	KF611911.1
P4-9	GGGCATCGCATGGAGAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAG GGAAGAACAAGTGCGAGAGTAACTGC TCGCACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. subtilis*	99	KC595863.1
P5-6	GTAATTTCGCATGGAGAAGTCTGACG GAGCACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAACTCTGTTGTTAG GGAAGAACAAGTACCGTTCGAATAGG GCGGTACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAAT	*B. subtilis*	99	GU434356.1
P7-1	GGGGCTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAACTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATT	*B. licheniformis*	99	MF527241.1
P7-2	GGGCTCGCATGGAGAAGTCTGACGGA GCACGCCGCGTGAGTGATGAAGGTTT TCGGATCGTAAAACTCTGTTGTTAGGG AAGAACAAGTACCGTTCGAATAGGGC	*B. licheniformis*	99	MF527241.1

	GGTACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATC			
P7-5	GGCATGGCATGGACGGAGTCTTGACT GAGCCCTGCCGGCGTGATGTGATAGA GGTTTTCTTGATCTGAAAGCTCTGTTG TTAGGGAAGAACACCTGCCGGAGTAT CTGCGTGCACCTTGACGGTACCTAACC AAAAAGCCCCGGCTAACTCTGTGCCA GCAGCCGCGGTAATTA	*B. altitudinis*	85	HQ849482.1
P8-1	GGGCTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTGCAAGAGTAACTGCT TGCACCTTGACGGTACCTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATTGA	*B. pumilus*	99	HM055957.1
P4-2	GGCGTGTAATTCTGGTAGGTACGTCA GGTGCCGCCCTATTTGAACGGCACTTG TTCTTCCCTAACAACAGAGCTTTACGA TCCGAAAACCTTCATCACTCACGCGG CGTTGCTCCGTCAGACTTTCGTCCATT GCGGAAGATTCCCTACTGCTGCCTCCC GTAGG	*B. atrophaeus*	99	KU955683.1
P8-2	GGACTTCGCATGGAGAAGTCTGACGG AGCACGCCGCGTGAGTGATGAAGGTT TTCGGATCGTAAAGCTCTGTTGTTAGG GAAGAACAAGTACCGTTCGAATAGGG CGGTACCTTGACGGTACCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATGA	*B. subtilis*	99	EU532192.1
P14-1	GGCATGGCATGGACGGAGTCTTGACT GAGCCCTGCCGGCGTGATGTGATAGA GGTTTTCTTGATCTGAAAGCTCTGTTG TTAGGGAAGAACACCTGCCGGAGTAT CTGCGTGCACCTTGACGGTACCTAACC AAAAAGCCCCGGCTAACTCTGTGCCA GCAGCCGCGGTAATTA	*B. altitudinis*	98	HQ849482.1

APPENDIX D7

1.5% agarose gel showing bacterial DNA (193bp)



APPENDIX D8

Protective culture application in cheese sample

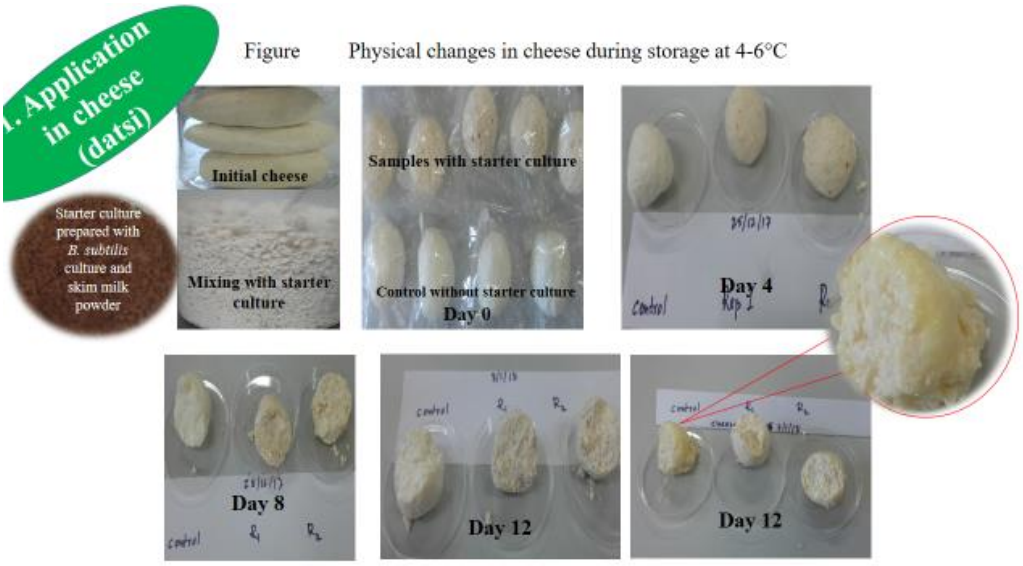


Figure Physical changes in cheese during storage at 4-6°C

APPENDIX D9

Protective culture application in bamboo shoot pickle

Figure Physical changes in bamboo shoot pickle during storage at RT

APPENDIX D10
Caco2 cytotoxicity result

Bioassay laboratory *TEST REPORT*

BIOTEC a member of NSTDA

Customer name: พิชญาภา คณานุรักษ์
Customer Address : ภาควิชาเทคโนโลยีทางอาหาร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Test: Cytotoxicity against human caucasian colon adenocarcinoma (Caco2) ATCC HTB-37
Method: Resazurin Microplate assay (REMA)
IC_{50} of positive control: Ellipticine = 14.42 µg/ml
Reported date (dd/mm/yy): 08/02/2018
Total No. of tested sample: 1

Item	Screening code	Sample code	Final concentration (µg/ml)	Fluorescence unit		% Cytotoxicity	Activity	IC_{50} (µg/ml)
				Average	SD			
	Negative	Cell+DMSO	1% DMSO	22627	1187	0.00	-	-
	Positive1	Ellipticine	40.00	7768	615	65.67	Cytotoxic	
			20.00	10318	839	54.40	Cytotoxic	14.42
			10.00	12747	365	43.67	Non-cytotoxic	-
			5.00	17911	2161	20.84	Non-cytotoxic	-
			2.50	18431	1997	18.54	Non-cytotoxic	-
			1.25	20054	971	11.37	Non-cytotoxic	-
	Negative	Cell+DW	Distilled water	24379	1786	0.00	-	-
1	VA1436	สารสกัดจากจุลินทรีย์	100.00	20850	838	14.48	Non-cytotoxic	-
			50.00	23156	613	5.02	Non-cytotoxic	-
			25.00	22469	1554	7.84	Non-cytotoxic	-
			12.50	22315	2061	8.47	Non-cytotoxic	-
			6.25	23107	1413	5.22	Non-cytotoxic	-
			3.13	22915	594	6.01	Non-cytotoxic	-

Remark:

Assayed by _____
(Pattiyaa Laksanacharoen)
(08/02/18)

Approved by _____
(Kannawat Danwisetkanjana)
(09/02/18)

% Cytotoxicity
< 50%
≥ 50%

Activity
Non-cytotoxic
Cytotoxic (IC_{50} included)

National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA)
113 Paholyothin Rd, Klong Nueng, Klong Luang, Pathumthani 12120, Thailand
Tel. 02-5646629, Fax 02-5646707, www.biotec.or.th/bioassay

APPENDIX D11
Vero cytotoxicity result

Bioassay laboratory *TEST REPORT*

BIOTEC
a member of NSTDA

Customer name: พิชญาภา คณานุรักษ์
Customer Address : ภาควิชาเทคโนโลยีทางอาหาร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Test: Cytotoxicity against Vero cells (African green monkey kidney)
Method: Green Fluorescent Protein (GFP)-based assay
IC_{50} of positive control: Ellipticine = 1.28 µg/ml
Reported date (dd/mm/yy): 17/01/2018
Total number of sample: 1

Item	Screening code	Sample code	Final concentration (µg/ml)	Fluorescence unit at Day0		Fluorescence unit at Day4		% Cytotoxicity	Activity	IC_{50} (µg/ml)
				Average	SD	Average	SD			
	Negative	Cell+DMSO	0.5% DMSO	1372	41	2718	114	0.00	-	-
	Positive	Ellipticine	4.00	1454	37	1772	91	76.39	Cytotoxic	
			2.00	1397	52	1989	101	56.05	Cytotoxic	1.28
			1.00	1410	35	2104	81	48.49	Non-cytotoxic	
			0.50	1388	45	2283	87	33.47	Non-cytotoxic	
			0.25	1392	31	2473	69	19.71	Non-cytotoxic	
			0.13	1392	39	2547	65	14.18	Non-cytotoxic	
	Negative	Cell+DW	Distilled water	1395	33	3562	115	0.00	-	-
1	VA1436	สารสกัดจากจุลินทรีย์	50.00	1399	37	5229	155	-76.72	Non-cytotoxic	-
			16.67	1348	21	4797	147	-59.16	Non-cytotoxic	-
			5.56	1351	34	4483	80	-44.56	Non-cytotoxic	-
			1.85	1374	51	4083	208	-24.99	Non-cytotoxic	-
			0.62	1375	55	3759	89	-10.02	Non-cytotoxic	-
			0.21	1371	58	3656	48	-5.44	Non-cytotoxic	-

Remark:

Assayed by Kitlada S. (Kitlada Srichomthong) (17 / 01 / 18)

Approved by Kannawat D. (Kannawat Danwisetkanjana) (17 / 01 / 18)

Interpretation

% Cytotoxicity	Activity
< 50%	Non-cytotoxic
≥ 50%	Cytotoxic (IC_{50} included)

National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA)
113 Phaholyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand
Tel. 02-5646629, Fax 02-5646707, Email: bioassayservice@biotec.or.th, www.biotec.or.th/bioassay

APPENDIX D12
Acute toxicity result

Medicinal Plant Research Institute,
Department of Medical Sciences, Ministry of Public Health,
Tiwanon Road, Amphur Muang, Nonthaburi Province. 11000 Thailand
Tel. (66) 2589 0022, (66) 2951 0000 Ext 99963 Fax. (66) 2589 9866
Accreditation No. 1160/52

ANALYTICAL REPORT

Sample No.	19-61-008644-001	Report No.	019 /2018
Sample Name	Cottage cheese mixed antimicrobial extract	Received Date	29 November 2017
Analyzed Date	6 February 2018	Reported Date	21 February 2018
Customer	Miss Phichayapha Kananurak Faculty of Science Chulalongkorn University Payathai Road, Wangmai, Pathumwan Bangkok 10330 Tel. 084-259-6682		
Description & Packaging	Yellow coarse powder which is contained in sealed aluminum foil. There is labeled sample Cottage cheese+starter culture (Skim milk powder+*Bacillus subtilis* 100 g 28/11/17 4 °C)		
Quantity	100 g 6 bags		
Objective	Acute toxicity test		
Result	Sample was suspended in distilled water and adjusted to the concentration of 0.25 g/ml. The sample suspension was orally administered to mice at the volume of 20 ml/kg. Acute toxicity test result revealed that the experimental group had no abnormal signs after receiving the sample. All experimental mice survived till the end of the experiment. Necropsy revealed no gross lesions in the visceral organs when compared with control group. Therefore, LD_{50} of the sample should be more than 5.0 g/kg. The toxicity category of sample was classified as Category 5		
Analysis Method	SOP 12 02 004 base on standard method for acute oral toxicity by OECD (Guideline 423), 2001		

Signature Praw Suppajariyawat Reporter
(Miss Praw Suppajariyawat)
Medical Scientist, Professional Level

Signature Pornchai Sincharoenpokai Approver
(Mr. Pornchai Sincharoenpokai)
Lab supervisor

VITA

Mr. Vishal Chhetri is born on 8th April 1985 in Paro, Bhutan. He graduated with Bachelor Degree in the field of Allied Health Science with the specialization in clinical laboratory in 2010, from Sri Ramachandra Medical and Research Institute (SRMC&RI) Chennai, South India. He has been working as the Medical Technologist (PM1167) at the Gelephu Central Regional Referral Hospital (GCRRH), Sarpang, Bhutan from 2011, until he joined Chulalongkorn University in 2016 to pursue his further study. So, he has been a graduate student in the Master's Degree in Food Science and Technology program, Faculty of science, Chulalongkorn University since January 2016.

Publication from this thesis

1. Halophilic bacteria from salty fermented foods as potential bio-preservative against Staphylococcus aureus contamination in foods. The 19th Food Innovation Asia Conference 2017 (FIAC 2017) 15-17 June 2017. Proceeding

2. Poster presentation: Bacillus; Bacteriocin producers as biocontrol agents for Staphylococcus aureus. ASM Microbe 2018, June 7-11, 2018 Atlanta, USA. Control No. 2142

3. Article in preparation: Halophilic Bacillus from Plara and bacteriocin encoding gene expression to use as bio-control agent against Staphylococcus aureus in salt added foods