Molecular Approaches for Microbial Diversity, Screening, Characterization of Bacteriocin Producing Lactic Acid Bacteria and Its Application in Fermented Shrimp (*Kung-Som*) Production

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Thesis Title	Molecular Approaches for Microbial Diversity, Screening, Characterization of Bacteriocin Producing Lactic Acid Bacteria and Its Application in Fermented Shrimp (*Kung-Som*) Production
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ชื่อวิทยานิพนธ์ การใช้เทคนิคทางชีวโมเลกุลเพื่อศึกษาความหลากหลายของจุลินทรีย์ การคัดเลือก คุณลักษณะของแบคทีเรียแลกติกที่ผลิตแบคเทอริโอซินและการประยุกต์ใช้ในการผลิตกุ้งส้ม

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บทคัดย่อ

กุ้งส้มเป็นอาหารหมักชนิดหนึ่งที่นิยมบริโภคกันอย่างแพร่หลายในภาคใต้ของประเทศไทย วัตถุดิบในการผลิตกุ้งส้มคือ กุ้งทะเลขนาดเล็ก (*Fenneropenaeus merguiensis*) น้ำตาล เกลือ และน้ำ จากนั้นหมักด้วยวิธีธรรมชาติโดยอาศัยแบคทีเรียประจำถิ่นในวัตถุดิบ ส่งผลให้กุ้งส้มมีกลิ่นและรสชาติที่เป็นเอกลักษณ์ การศึกษาครั้งนี้ทำการตรวจสอบความปลอดภัยทางจุลชีววิทยาของกุ้งส้มที่จำหน่ายในท้องตลาดและศึกษาความหลากหลายของแบคทีเรียในตัวอย่างกุ้งส้มด้วยเทคนิค polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) พบว่าในทุกตัวอย่างของกุ้งส้มตรวจไม่พบเชื้อก่อโรค ได้แก่ *Escherichia coli, Clostridium perfringens, Staphylococcus aureus* และ *Salmonella* sp. นอกจากนี้ผลการวิเคราะห์ความหลากหลายของกลุ่มประชากรแบคทีเรียในกุ้งส้ม โดยใช้ส่วนของ V3 บนยีน 16S rRNA พบว่าแบคทีเรียแลกติกเป็นกลุ่มประชากรหลักในผลิตภัณฑ์กุ้งส้ม ได้แก่ *Lactobacillus farciminis*, *Lb. plantarum*, *Lactococcus garvieae*, *Tetragenococcus halophillus* และ *Weissella thailandensis* นอกจากนี้ผลการจำแนกความหลากหลายของกลุ่มประชากรแบคทีเรียด้วยเทคนิค DGGE พบว่าแบคทีเรียกลุ่ม coagulase-negative staphylococci (CNS) เป็นแบคทีเรียกลุ่มรองในผลิตภัณฑ์กุ้งส้ม จากผลการศึกษาทำให้ทราบถึงความหลากหลายของกลุ่มประชากรแบคทีเรียเพื่อพัฒนาเป็นกล้าเชื้อในการผลิตกุ้งส้ม

ปัจจุบันมีการใช้แบคเทอริโอซินที่ผลิตโดยแบคทีเรียแลกติกอย่างแพร่หลายเพื่อใช้เป็นสารชีวภาพที่ออกฤทธิ์ยับยั้งแบคทีเรียแทนที่ยาปฏิชีวนะในอุตสาหกรรมอาหาร แบคทีเรียแลกติกสายพันธุ์ *Lactococcus lactis* KTH0-1S ที่แยกได้จากกุ้งส้มสามารถผลิตแบคเทอริโอซินที่ทนร้อนและมีความสามารถยับยั้งแบคทีเรียที่ก่อโรคในอาหารและแบคทีเรียที่ทำให้อาหารเน่าเสีย การศึกษาคุณสมบัติของแบคเทอริโอซิน พบว่าแบคเทอริโอซินมีความคงตัวที่พีเอช 2-11 และอุณหภูมิ 80-121°C แต่กิจกรรมการยับยั้งของแบคเทอริโอซินสูญเสียไปเมื่อถูกย่อยด้วยเอนไซม์กลุ่มโปรติเอส การศึกษากิจกรรมการยับยั้งของ *Lc. lactis* KTH0-1S ต่อแบคทีเรียก่อโรค *S. aureus* พบว่า *Lc. lactis* KTH0-1S สามารถยับยั้งการเจริญของ *S. aureus* อย่างมีนัยสำคัญเมื่อเลี้ยงร่วมกัน

เป็นเวลา 9 ชั่วโมง นอกจากนี้การเติมแบคเทอริโอซิน KTH0-1S ร่วมกับการเลี้ยงแบคทีเรีย *S. aureus* พบว่าจำนวนเซลล์ *S. aureus* ลดลงอย่างรวดเร็ว (2 log CFU/ml) ซึ่งแสดงให้เห็นว่าแบคเทอริโอซินมีกิจกกรมการยับยั้งแบบ bactericidal ภายหลังการทำบริสุทธิ์แบคเทอริโอซิน KTH0-1S ซึ่งประกอบด้วย 4 ขั้นตอน โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต, reverse phase cartridge (C_8 Sep-Pak), โครมาโตกราฟีแบบแลกเปลี่ยนประจุบวก และ RP-HPLC (C_8 column) เมื่อนำแบคเทอริโอซินบริสุทธิ์ KTH0-1S ไปวิเคราะห์ด้วยเครื่อง mass spectrometry พบว่าแบคเทอริโอซิน KTH0-1S มีน้ำหนักโมเลกุลเท่ากับ 3.346 กิโลดาลตัน และเมื่อศึกษาหายีนไนซินด้วยเทคนิค PCR โดยใช้ไพรเมอร์ที่มีความจำเพาะเจาะจงและวิเคราะห์ลำดับนิวคลีโอไทด์ พบว่าแบคเทอริโอซินที่ผลิตโดย *Lc. lactis* KTH0-1S คือ ไนซิน Z (nisin Z) นอกจากนี้การศึกษาความปลอดภัยของ *Lc. lactis* KTH0-1S ไม่พบการดื้อต่อยาปฏิชีวนะ ไม่ผลิตสารกลุ่มไบโอจีนิก-เอมีน (non-biogenic amines) และไม่พบ virulence factors gene และ ผลการศึกษาสรุปได้ว่าแบคทีเรียแลกติก *Lc. lactis* KTH0-1S มีศักยภาพในการใช้เป็นกล้าเชื้อเพื่อผลิตกุ้งส้มให้มีความปลอดภัยเพิ่มขึ้น

การศึกษาการลดปริมาณสารกลุ่มไบโอจีนิกเอมีนในอาหารหมัก พบว่าแบคทีเรียแลกติกที่ผลิตแบคเทอริโอซินสามารถยับยั้งการเกิดสารกลุ่มไบโอจีนิกเอมีนได้ ซึ่งไทรามีน (tyramine) เป็นสารในกลุ่มไบโอจีนิกเอมีนที่พบมากในอาหารหมักหลายชนิด มีความเป็นพิษต่อร่างกายมนุษย์ ไทรามีนเกิดจากการเปลี่ยนกรดอะมิโนไทโรซีน (tyrosine) โดยกิจกรรมของเอนไซม์ไทโรซีนดีคาร์บอกซิเลส (tyrosine decarboxylase) ของแบคทีเรียที่พบในอาหารหมัก การศึกษานี้ได้คัดเลือกแบคทีเรียที่มีความสามารถผลิตไทรามีนด้วยวิธีโครมาโตกราฟีและชีวโมเลกุล จากแบคทีเรีย 20 ไอโซเลตที่แยกจากกุ้งส้ม พบว่าตรวจพบยีน tyrosine decarboxylase (*tdc*) และการผลิตไทรามีนจากแบคทีเรีย 3 ไอโซเลต คือ *Enterococcus faecalis* D0KS13, *Ent. faecalis* 9Y และ *Weissella cibaria* D0KS11 เมื่อศึกษาผลการยับยั้งการเจริญของแบคทีเรียที่ผลิตไทรามีนด้วย *Lc. lactis* KTH0-1S ที่ผลิตไนซิน Z โดยวิธีเพาะเลี้ยงร่วมกัน พบว่า *Lc. lactis* KTH0-1S สามารถยับยั้งอัตราการเจริญและจำนวนเซลล์สูงสุดของ *Ent. faecalis* D0KS13 และ *W. cibaria* D0KS11 อย่างมีนัยสำคัญ ซึ่งผลการยับยั้งขึ้นกับจำนวนเซลล์เริ่มต้นของแบคทีเรียที่ผลิตไทรามีน นอกจากนี้ปริมาณไทรามีนสะสมจะลดลงอย่างรวดเร็วในชุดการทดลองที่มีจำนวนเซลล์เริ่มต้นของแบคทีเรียที่ผลิตไทรามีนเท่ากับ 4.0 log CFU/ml และลดช้าลงเมื่อจำนวนเซลล์เริ่มต้นของแบคทีเรียที่ผลิตไทรามีนเท่ากับ 5.0 และ 6.0 log CFU/ml นอกจากนี้ชุดการทดลองที่เติม *Lc. lactis* KTH0-1S เป็นกล้าเชื้อในการผลิตกุ้งส้มสามารถยับยั้งการเจริญของแบคทีเรียทั้งหมด แบคทีเรียกลุ่ม Enterobacteriacea และ *S. aureus* เมื่อเปรียบเทียบกับชุดควบคุมที่ไม่เติมกล้าเชื้อในการผลิตกุ้งส้ม

ส่วนปริมาณไทรามีนในกุ้งส้มที่เติม *Lc. lactis* KTH0-1S เป็นกล้าเชื้อสามารถลดไทรามีนลง 100 mg/kg เปรียบเทียบกับกุ้งส้มที่หมักโดยวิธีธรรมชาติ จากผลการศึกษานี้แสดงให้เห็นว่า *Lc. lactis* KTH0-1S เหมาะสมเป็นกล้าเชื้อในการผลิตกุ้งส้มเพื่อลดการปนเปื้อนเชื้อก่อโรคและปริมาณไทรามีน

จากผลการศึกษาความหลากหลายของกลุ่มประชากรแบคทีเรียด้วยวิธี PCR-DGGE พบว่าแบคทีเรียกลุ่ม CNS เป็นกลุ่มประชากรรองในกุ้งส้ม มีการใช้แบคทีเรียกลุ่ม CNS เป็นกล้าเชื้อในอาหารหมักหลากหลายชนิด เนื่องจากแบคทีเรียกลุ่มนี้มีผลต่อสีและคุณภาพด้านประสาทสัมผัสของอาหารหมัก การศึกษานี้มีวัตถุประสงค์เพื่อคัดเลือกแบคทีเรีย CNS ที่มีความปลอดภัยและคุณสมบัติที่ดีเพื่อใช้เป็นกล้าเชื้อในการผลิตกุ้งส้ม จากแบคทีเรียกลุ่ม CNS 46 ไอโซ-เลต ที่แยกจากกุ้งส้ม มีเพียง 1 ไอโซเลต คือ แบคทีเรียสายพันธุ์ *Staphylococcus carnosus* K21K22 ที่มีความปลอดภัย เนื่องจากไม่พบการดื้อยาปฏิชีวนะ ไม่พบการย่อยเม็ดเลือดแดง และไม่ผลิตสารกลุ่มไบโอจีนิกเอมีน นอกจากนี้แบคทีเรียสายพันธุ์นี้แสดงกิจกรรมของเอนไซม์ไลเปส โปรติเอส และไนเตรตรีดักเตส (nitrate reductase) และเจริญในสภาวะที่เป็นกรดและเกลือสูง นอกจากนี้การศึกษาเปรียบเทียบกุ้งส้มที่ผลิตโดยใช้กล้าเชื้อเดี่ยว *Lc. lactis* KTH0-1S และ *S. carnosus* K21S22 หรือกล้าเชื้อผสมระหว่าง *Lc. lactis* KTH0-1S และ *S. carnosus* K21S22 และการผลิตกุ้งส้มแบบดั้งเดิม พบความแตกต่างของพีเอชและปริมาณกรดทั้งหมดอย่างมีนัยสำคัญทางสถิติ (p<0.05) อย่างไรก็ตามไม่พบจุลินทรีย์ก่อโรค ได้แก่ *B. cereus*, *C. perfringens*, *E. coli*, *S. aureus* และ *Salmonella* sp. ในกุ้งส้ม ทั้ง 4 ชุดการทดลอง การศึกษาทางประสาทสัมผัสของกุ้งส้มทั้ง 4 ชุดการทดลอง ได้แก่ ชุดควบคุมที่ไม่เติมกล้าเชื้อ ชุดที่เติมเฉพาะกล้าเชื้อ *Lc. lactis* KTH0-1S ชุดที่เติมเฉพาะกล้าเชื้อ *S. carnosus* K21S22 และชุดที่เติมกล้าเชื้อผสมระหว่าง *Lc. lactis* KTH0-1S และ *S. carnosus* K21S22 พบว่าผู้ทดสอบชิมให้คะแนนความชอบลักษณะปรากฏ สี รสชาติ กลิ่นรส เนื้อสัมผัสและความชอบรวมของกุ้งส้มทั้ง 4 ชุดการทดลอง มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p<0.05) โดยผู้ทดสอบชิมให้ผลคะแนนความชอบด้านประสาทสัมผัสในกุ้งส้มที่ผลิตโดยใช้กล้าเชื้อ *Lc. lactis* KTH0-1S และกล้าเชื้อผสมระหว่าง *Lc. lactis* KTH0-1S และ *S. carnosus* K21S22 มากกว่ากุ้งส้มที่ผลิตแบบดั้งเดิมและกุ้งส้มที่เติมเฉพาะกล้าเชื้อ *S. carnosus* K21S22

จากการศึกษาทั้งหมดสามารถสรุปได้ว่าแบคทีเรียสายพันธุ์ *Lc. lactis* KTH0-1S เป็นกล้าเชื้อที่ดีในการผลิตกุ้งส้ม อีกทั้งการศึกษานี้ยังสามารถขยายองค์ความรู้เกี่ยวกับการประยุกต์ใช้แบคทีเรียแลกติกที่ผลิตแบคเทอริโอซินหรือแบคทีเรียกลุ่ม CNS เพื่อเป็นกล้าเชื้อในผลิตภัณฑ์อาหารหมักต่าง ๆ เพื่อลดระยะเวลาการหมัก เพิ่มความปลอดภัย ยืดอายุการเก็บรักษาและปรับปรุงคุณภาพของผลิตภัณฑ์

Thesis Title	Molecular Approaches for Microbial Diversity, Screening, Characterization of Bacteriocin Producing Lactic Acid Bacteria and Its Application in Fermented Shrimp (*Kung-Som*) Production
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ABSTRACT

Kung-som is a traditionally Thai fermented shrimp which is popularly consumed and distributed in the southern part of Thailand. It is produced by naturally fermentation of small sea shrimp (*Fenneropenaeus merguiensis*) mixing with sugar, sea salt and water, resulting in the unique characteristic of flavors and tastes. The aim of this study was to evaluate food safety of *kung-som* that distributed in local markets and to identify bacterial community of *kung-som* using polymerase chain reaction-denaturing gel gradient electrophoresis (PCR-DGGE) technique. For microbiological safety, all samples of *kung-som* did not find the pathogenic bacteria including *Escherichia coli*, *Clostridium perfringens*, *Staphylococcus aureus* and *Salmonella* sp. The bacterial DGGE profile targeting the V3 region of 16S rRNA gene indicated that the predominant lactic acid bacteria (LAB) belonged to *Lactobacillus farciminis*, *Lb. plantarum*, *Lactococcus garvieae*, *Tetragenococcus halophilus* and *Weissella thailandensis*. Additionally, DGGE result presented coagulase-negative staphylococci (CNS) as a minor microflora in *kung-som*. Our finding suggested that understanding of bacterial community presented in *kung-som* and providing a theoretical basis to develop the starter cultures for production of *kung-som*.

Presently, bacteriocin-producing LAB have been widely used as an alternative food-perseverative agent replacing the antibiotics in food-industry. *Lactococcus lactis* KTH0-1S strain isolated from Thai traditional fermented shrimp (*kung-som*) had ability to produce heat-stable bacteriocin and demonstrated the antagonistic activity against food-borne pathogens and food spoilage bacteria. An inhibitory activity of bacteriocin retained active after treatment with different pHs (2-11) and temperatures (80-121°C) but was sensitive to some proteolytic enzymes. The growth of *S. aureus* significantly decreased after 9 h co-cultivation with bacteriocin-

producing *Lc. lactis* KTH0-1S. Moreover, the addition of bacteriocin KTH0-1S to *S. aureus* cultures resulted in viable cell counts sharply decreased by 2.0 log CFU/ml demonstrating a bactericidal mode of action. After four steps of purification (ammonium sulfate precipitation, reverse phase cartridge (C_8 Sep-Pak), cation-exchange chromatography, RP-HPLC on C_8 column), the purified bacteriocin KTH0-1S had a molecular mass of 3.346 kDa using mass spectrometry (MS/MS) analysis. Bacteriocin KTH0-1S was identified as nisin Z by using PCR amplification and sequencing. Additionally, nisin Z-producing *Lc. lactis* KTH0-1S was sensible towards most of studied antibiotics, non-biogenic amines production and did not harbor virulence genes. Our finding concluded that *Lc. lactis* KTH0-1S presented a high potential to be used as a starter culture for improving a microbiological safety in *kung-som*.

In order to reduce the accumulation of biogenic amines in fermented foods, bacteriocin-producing LAB showing positive effect against biogenic amines formation. Tyramine as biogenic amine is most frequently found in various fermented foods that present toxicological effects on human. It occurred from tyraminogenic bacteria by conversion of tyrosine into tyramine through tyrosine decarboxylase activity. Tyramine production of 20 strains of LAB and CNS isolated from Thai traditional fermented shrimp (*kung-som*) was investigated by using chromatographic and molecular methods. Among 20 of bacterial strains isolated from *kung-som*, *Enterococcus faecalis* 9Y, *Ent. faecalis* D0KS13 and *Weissella cibaria* D0KS11 were able to produce tyramine and also possess the tyrosine decarboxylase (*tdc*) gene. The effectiveness of nisin Z-producing *Lc. lactis* KTH0-1S against tyraminogenic strains was evaluated in co-cultivation. In competition study, *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 decreased significantly the growth rate and the maximal cell concentration, depending on the initial inoculum level of tyraminogenic strain. The reduction of tyramine accumulation decreased strongly when tyraminogenic strain was inoculated at 4.0 log CFU/ml and, to a lesser extent, at 5.0 and 6.0 log CFU/ml. Additionally, *kung-som* inoculation with nisin Z-producing *Lc. lactis* KTH0-1S as a starter culture could suppress the growth of undesirable bacteria such as Enterobacteriaceae, *S. aureus* and total viable count (TVC), comparing with spontaneous fermented *kung-som* (without starter culture). The addition of *Lc. lactis* KTH0-1S as a starter culture in *kung-som* reduced accumulation of tyramine about 100

mg/kg in comparison with naturally fermented *kung-som*. Our result indicated that *Lc. lactis* KTH0-1S as a starter culture is advisable for production of safer *kung-som* with decreasing pathogen contaminants and lowering the tyramine content.

Furthermore, the obtained result from DGGE profile showed the coagulase-negative staphylococci (CNS) as minor bacterial flora in *kung-som*. CNS have been used as starter culture in various fermented foods, they play a major role in the development of sensory properties and color development of fermented foods. The safety evaluation and technological characteristics were primary criteria for selection of CNS to be applied as starter culture in *kung-som* production. Among 46 CNS strains, only strain *Staphylococcus carnosus* K21S22 was considered as safe to be used with regard to its sensitiveness to antibiotics tested, non-hemolytic and non-biogenic production. Additionally, its strain showed the lipolytic, proteolytic and nitrate reductase activities, as well as its adaptability under high salt content and acidic conditions. Comparison of *kung-som* production by using single starter culture (*S. carnosus* K21S22 or *Lc. lactis* KTH0-1S), mixed starter cultures and spontaneous *kung-som*, they were significant differences ($p<0.05$) in pH, titratable acidity. However, the pathogenic bacteria such as *B. cereus*, *C. perfringens*, *E. coli*, *S. aureus* and *Salmonella* sp. did not detect in all *kung-som* treatments. For sensory evaluation, all *kung-som* treatments (spontaneous, inoculated with only *Lc. lactis* KTH0-1S or *S. carnosus* K21S22 and mixed starter cultures) were significant difference ($p<0.05$) in appearance, taste, texture, flavor and overall acceptance. The panelists gave the higher score for organoleptic of *kung-som* inoculated with *Lc. lactis* KTH0-1S and mixed culture than the spontaneous *kung-som* and inoculated *kung-som* with *S. carnosus* K21S22.

Regarding to our results obtained, it indicates that nisin Z-producing *Lc. lactis* KTH0-1S are good promising starter culture to successfully apply in *kung-som* fermentation. It will further expand the application area of bacteriocin producing LAB or CNS strain as starter culture to fermented products for shorten time periods of fermentation, increasing food safety, extended shelf-life and improving quality of products.

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LIST OF ABBREVIATION AND SYMBOL

A_{595}	=	Absorbance at wavelength 595 nanometer
ACN	=	Acetonitrile
AU	=	Arbitrary unit
BAs	=	Biogenic amines
BHI	=	Brain heart infusion
CFS	=	Cell free supernatant
CFU	=	Colony forming unit
CNC	=	Coagulase-negative cocci
CNS	=	Coagulase-negative staphylococci
DGGE	=	Denaturing gradient gel electrophoresis
DNA	=	Deoxyribonucleic acid
DnsCl	=	Dansyl chloride
EDTA		Ethylenediaminetetraacetic acid
EFSA	=	European Food Safety Authority
EUCAST	=	European Committee on Antimicrobial Susceptibility Testing
FAO	=	Food and Agricultural Organization
FDA	=	Food and Drug Administration
GC-MS	=	Gas chromatography-mass spectrometry
GRAS	=	Generally Recognized As Safe
hdc gene	=	Histidine decarboxylase gene
HS-SPME	=	Head space-solid phase microextraction
icaA gene	=	Intercellular adhesion gene
IsOH	=	Isopropanol
kDa	=	Kilodalton
LAB	=	Lactic acid bacteria
ldc gene	=	Lysine decarboxylase gene
MIC	=	Minimum inhibitory concentration
MPN	=	Most probable number
MRS	=	de Man-Rogosa-Sharpe
MRSA	=	Methicillin resistance *Staphylococcus aureus*

LIST OF ABBTREVIATIION AND SYMBOL (Cont.)

MS/MS	=	Mass spectrometry/mass spectrometry or Tandem mass spectrometry
MSA	=	Mannitol salt agar
MW	=	Molecular weight
NCFS	=	Neutralized cell free supernatant
OD_{600}	=	Optical density at wavelength 600 nanometer
odc gene	=	Ornithine decarboxylase gene
PBS	=	Phosphate buffer
PCA	=	Plate count agar
PCR	=	Polymerase chain reaction
PDA	=	Potato dextrose agar
PPB	=	Partial purified bacteriocin
PTFE	=	Polytetrafluoroethylene
qPCR	=	Qualitative polymerase chain reaction
QPS	=	Qualified Presumption of Safety
RNA	=	Ribonucleic acid
RP-HPLC	=	Reverse phase-high performance liquid chromatography
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	=	Staphylococcus enterotoxin
TCA	=	Trichloroacetic acid
TCPS	=	Thai community product standard
tdc gene	=	Tyrosine decarboxylase gene
TFA	=	Trifluoroacetic acid
TLC	=	Thin layer chromatography
TSA	=	Trypticase soy agar
TSB	=	Trypticase soy broth
TVC	=	Total viable count
VGRB	=	Violet red bile agar
VRE	=	Vancomycin resistance enterococci
WHO	=	World Health Organization

CHAPTER 1

INTRODUCTON AND REVIEW OF LITERATURE

Introduction

Nowadays, consumer awareness of the effect of diet on health has led to a demand for minimally processed foods in which chemical preservatives are replaced by more natural alternatives. Traditionally foods were preserved by lactic acid bacteria (LAB), natural constituents of fermented foods which confer their preservative effects by the production of lactic acid, hydrogen peroxide as well as small peptides known as bacteriocins. Bacteriocins are active peptide against a number of genera (broad spectrum) or particular species (narrow spectrum) (Katikou *et al*., 2005; Nes *et al*., 2007; Barbosa *et al*., 2014) and are very diverse, varying in size, structure and specificity. The fact that many bacteriocins are produced by food-grade LAB and possess potent antimicrobial activity means that they are ideally suited to controlling food spoilage and pathogenic bacteria (Gálvez *et al*., 2010; Snyder and Worobo, 2014). They main advantage over chemical preservatives is their ability to preserve without several side-effects such as not change in sensory qualities of food and non-toxigenic on the human health (Sharma *et al*., 2006).

The possible application of bacteriocin-producing LAB as starter culture has been tested in dairy products (Tabanelli *et al*., 2014; Bockelmann *et al*., 2017), in fermented sausage (Lu *et al*., 2015), in fermented fish (Zhang *et al*., 2013) and in other food products (Khan and Oh, 2016) Moreover, the inoculation of bacteriocinogenic LAB as starter culture has been tested successfully in fermented products to prevent excessive biogenic amines (BAs) accumulation (Tosukhowong *et al*., 2011; Zhang *et al*., 2013; Kongkiattikajorn, 2015; Sun *et al*., 2016). For fermentation processes require a bacteriocinogenic strain well adapted to the particular food environment, in which it will be used, that is able to grow under the food processing and/or storage conditions and which produce bacteriocin in sufficient amounts to inhibit the target bacteria. LAB originally isolated from certain food products could be the best choice as starter cultures for the same products because they may be more competitive than LAB from other sources (Trias *et al*., 2008). Although, LAB are considered as Generally Recognized As Safe (GRAS) and Qualified Presumption of Safety (QPS) status (EFSA, 2011b), several studies have been reported that LAB strains in food-associated were resistant to

the clinically antibiotics and existent virulence genes (Flórez *et al*., 2007; Perin *et al*., 2014). These mobile genetic elements can potentially be transferred to human or animal bacterial flora, including pathogens (Van Reenen and Dicks, 2011; Huddleston, 2014). These bacteria have to characterization to ensure the inexistence of acquired risk factors so that these would be safe for human and animal consumption (Zycka-Krzesinska *et al*., 2015).

In Thailand, there are many traditional fermented products which are consumed without heating after appropriate fermentation (pH lower than 4.6). Among them, *kung-som* is a Thai traditional fermented shrimp that is widely consumed due to its unique texture, flavor, and color. The basic ingredients of *kung-som* are small shrimp (*Fenneropenaeus merguiensis*), sugar and salt. Fermentation of *kung-som* involves the successive growth of different microorganisms, dominated by LAB naturally present in raw materials. *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Tetragenococcus* and *Weissella* have been identified as the predominant bacteria in *kung-som* fermentations (Sanchart *et al*., 2015; Saelao *et al*., 2016; Thongruck *et al*., 2017). However, *kung-som* produced by spontaneous fermentation, often result in fermentation failure, inconsistent qualities, and unsafe products by contamination of food-spoilage and pathogenic bacteria.

Additionally, the fermented shrimps or shrimp by products have been reported to be rich in essential amino acids (Sánchez-Machado *et al*., 2008) as a precursor of the biogenic amines (BAs), which can reach concentrations over the level of standard constituting an obvious health hazard. The relevant BAs in food and fermented food are cadaverine, histamine, putrescine and tyramine, which are occur by microbial decarboxylation of precursor amino acids such as lysine, histidine ornithine and tyrosine, respectively. Among them, histamine and tyramine are considered as the most toxic. European Food Safety Authority (EFSA, 2011a) reported that the high level of 50 mg/kg of histamine and 600 mg/kg of tyramine in foods exerts toxic effects on human health. Bacteriocin-producing LAB used as starter culture that was feasible alternative way to be reduced BAs content in fermented products as reported by Tosukhowong *et al*. (2011) and Sun *et al*. (2016). Even though, the addition of starter culture in *kung-som* production would guarantee obtaining products with repeatable hygienic and organoleptic properties in a shorter fermenting time (Sanchart *et al*., 2017). Nevertheless, *kung-som* was inoculated the only of LAB starter culture that

showed the organoleptic scores lower than those of spontaneous *kung-som* (Hwanhlem *et al*., 2010; Sanchart *et al*., 2017) because a diversity of microorganisms contribute to the development of a unique characteristic in the former. Previous study reported that coagulase-negative staphylococci (CNS) were bacterial flora in *kung-som* that might be contributed the organoleptic quality in this product (Sanchart *et al*., 2015; Thongruck *et al*., 2017). Several publications suggested that CNS strains, rather than LAB, play an important role to develop the organoleptic properties of fermented products through their metabolic activities (Casaburi *et al*., 2005; Martín *et al*., 2007; Müller *et al*., 2016; Sánchez Mainar *et al*., 2017).

Consequently, in this study we focused on isolation, screening and purification of the bacteriocin-producing LAB from *kung-som*. The effectiveness of reduction of BAs accumulation *in vitro* by selected bacteriocin-producing strain and safety characteristics of selected bacteriocin-producing LAB were studied for its application as starter culture and/or protective culture in *kung-som*. Additionally, the safety evaluation and technological properties in CNS isolated from *kung-som* were tested before application as starter culture in this product. Moreover, the effect of selected bacteriocin-producing LAB cooperated with selected CNS strain on microbiological properties, pH and sensory quality during the fermentation of *kung-som* was also investigated.

Review of Literature

1. *Kung-som*

Kung-som is a traditionally Thai fermented shrimp that is considered as a delicacy in the south of Thailand, where is well known for its fishy flavor, together with sour, salty and sweet taste. *Kung-som* is made by shrimp (*Fenneropenaeus merguiensis*), sugar and sea salt to depend on different traditional recipes and is fermented by the naturally occurring microbial for 7-20 days at room temperature until completion. This finished product showed an orange appearance due to the acidification by various LAB and coagulase-negative cocci (CNC) present in the raw material (Hwanhlem *et al*., 2010; Sanchart *et al*., 2015). Normally, *kung-som* produced by traditional method to be concern about its safety because of the unpredictable pre- and post-processing contamination by pathogenic bacteria that was harmful for human health's. For solving these problems, the use of LAB starter culture for *kung-som* fermentation would be ensured the obtaining product with consistency in a hygiene, organoleptic qualities in a shorter time as well as also improved the stability and the shelf-life of products (Sanchart *et al*., 2017).

The Thai Community Product Standard (TCPS, 2014) has been published a microbiological and chemical standard for *kung-som* product as follow. *Clostridium perfringens* and *Salmonella* spp. as food-borne pathogenic bacteria are not detected in sample. Besides, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* are unpermitted up to the level of 3 MPN/g, 100 CFU/g and 1,000 CFU/g, respectively. Yeast and molds detected less than 1000 CFU/g. For the chemical properties, the pH value of final product must be lower than 4.6.

2. Role of lactic acid bacteria in various fermented foods

The fermentation is one of the oldest processing of food preservation. The process driven by adventitious microbiota from the raw materials and in environment. LAB are involved in many fermentation processes of traditional fermented food, revealing their profound effects on improving nutritional, food qualities and safety (Talon *et al*., 2007; Prpich *et al*., 2015; Jampaphaeng *et al*., 2017). Several studies found that *Enterococcus*, *Leuconostoc Lactobacillus*, *Pediococcus* and *Weissella* genera predominated in various plant materials depending on the species of plants (vegetables) (Di Cagno *et al*., 2013; Moon *et al*., 2018). *Leuconostoc* strains are

heterofermentative LAB, they are able to generate CO_2, ethanol, and mannitol, and less acid production, which responded the high sensory quality of fermented vegetables (Di Cagno *et al*., 2013; Lee *et al*., 2018; Moon *et al*., 2018). For Thai traditional fermented foods including *Pla-ra*, *Pla-chom*, *Pla-som*, *Kung-chom*, *Som-fak* and *Hoi-dong* are fermented by *Enterococcus faecalis*, *Lactobacillus farciminis* and *Lb. plantarum* (Tanasupawat, 2009; Kopermsub and Yunchalard, 2010; Hwanhlem *et al*., 2011). These strains are acid-tolerant LAB that able to grow in low pH condition, which were detected in final stage of fermentation process (Kopermsub and Yunchalard, 2010). The acidification by LAB was an increase firmness and cohesiveness of the fermented meat products. The organic acids enhance pink color of fermented meat during fermentation process (Ammor and Mayo, 2007; Castro *et al*., 2011). Additionally, organic acid produced by LAB that inhibited food-borne pathogenic and spoilage bacteria in fermented products for improving shelf-life and food safety. For cereal beverages production, LAB was an increase the nutrient availability and reduce anti-nutrient compounds (oxalic acids and phytates) during fermentation process. Furthermore, the organoleptic quality of beverages depended on LAB strains (Nsogning Dongmo *et al*., 2017). Salmerón *et al*. (2015) reported that the malt-based beverages inoculated with *Lb. plantarum* and those with *Lb. acidophilus* and *Lb. reuteri* showed significant differences in the acetaldehyde content and sensory acceptance. Cereal-based beverage formulated with *Lb. plantarum* Lp. 758 that demonstrated the highest of key aroma compounds when comparing with control treatment (Nsogning Dongmo *et al*., 2017), suggesting, the suitable LAB strain may improve the quality of cereal-based beverages and sensory acceptance.

3. Lactic acid bacteria (LAB)

LAB comprise a divers group of Gram-positive, non-spore forming bacteria. They occur as cocci or rods, microaerophilic or facultatively anaerobic, lacking off cytochroms and catalase, producing lactic acid as the major end product during the fermentation of carbohydrates (at least 50%) and characterized by a GC content less than 50 mol%. Recently, LAB associated with food stuffs including species of genera: *Carnobacterium, Enterococcus*, *Lactobacillus*, *Lactococcus, Leuconostoc, Pediococcus, Oenococcus*, *Tetragenococcus, Streptococcus, Vagococcus* and *Weissella*. LAB are mainly mesophile to be able grow in the range 5-45°C (with a few

thermophilic strains) and survive in low pH condition, however, they are fastidious microorganism with requirement of highly nutrients for growth condition (Holzapfel, 2002; Shi *et al*., 2012; Liu *et al*., 2016; Özcelik *et al*., 2016). Member of the LAB can be classified into two different groups based on their carbohydrate metabolism such as homofermentative and heterofermentative LAB. The homofermentative LAB can metabolize glucose in glycolysis to generate two molecules of lactic acid (Embden-Meyerhof-Parnas pathway). This group composes of *Enterococcus, Lactococcus, Pediococcus, Streptococcus* and some lactobacilli. Contrary, the heterofermentative LAB can be produced lactate, CO_2 and ethanol from carbohydrate fermentation. Members of this group included *Leuconostoc* and *Weissella* genera and some strains of lactobacilli (Moon *et al*., 2018).

LAB are considered as Generally Recognized As Safe (GRAS) because their ubiquitous appearance in foods and their contribution to create a healthy environment in the human intestine (Hanchi *et al*., 2014; Leite *et al*., 2015). Many studies have been isolated LAB from the fermented foods, including fermented meat, fermented shrimp, fermented fish (Fontana *et al*., 2005; Hwanhlem *et al*., 2011; Woraprayote *et al*., 2016), vegetables, fruits, cereal-based beverages (Di Cagno *et al*., 2013; Nsogning Dongmo *et al*., 2017) and dairy products (cheese and yogurt) (Mahmoodi, 2010; Winkelströter *et al*., 2015). LAB play an important role in fermented food and food preservation, either as the natural microflora or as starter cultures added under controlled conditions. Several researchers used LAB as protective cultures and natural preservation in food stuffs because of they are a powerful inhibition of contaminated spoilage microorganisms, by producing various antimicrobial compounds such as acids, diacetyl, carbon dioxide (CO_2), hydrogen peroxide (H_2O_2), reuterin as well as bacteriocins (Cleveland *et al*., 2001; Nes and Johnsborg, 2004; Gálvez *et al*., 2007).

4. Antimicrobial substance produced by lactic acid bacteria

LAB fermented plants, meat, seafood and dairy products to preserve the nutritive qualities and improve shelf-life. The antimicrobial compounds are produced by LAB including organic acids, low-molecular-mass compounds (CO_2, diacetyl, H_2O_2 and reuterin) and high-molecular weight compounds like bacteriocins (Cleveland *et al*.,

2001; Nes and Johnsborg, 2004; Gálvez *et al*., 2007). These compounds have antagonistic activity against pathogenic and spoilage bacteria in food products.

4.1 Organic acids

The organic acids produced by LAB during the fermentation depending on species or strains, media composition and growth conditions. The inhibitory effect of organic acids is the reduction of pH level (Nes and Johnsborg, 2004; Gálvez *et al*., 2007). It has been proposed that the undissociated acid form can be inactivated the function of cell membrane. The undissociated acid acts by destroying the electrochemical proton gradient, and/or by collapsing the permeability of cell membrane. Lactic acid is the major organic acid to produce by LAB fermentation, its undissociated and dissociated forms were the dependent of pH (Cotter and Hill, 2003). Additionally, acetic acid showed the strongly inhibition activity against yeast and molds (Selwet, 2008; Özcelik *et al*., 2016).

4.2 Hydrogen peroxide (H_2O_2)

LAB produced H_2O_2 by the activity of flavoprotein-oxidase, NADH oxidase and superoxide dismutase under aerobic condition. The accumulation of H_2O_2 was significantly amounts *in vivo* because it is decomposed by peroxidase, flavoproteins and pseudo-catalase. The inhibitory effect of H_2O_2 caused by inactivating function of enzyme and dysfunction of membrane permeability. H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl (OH^-) radicals that can damage DNA (Boateng *et al*., 2011).

4.3 Carbon dioxide (CO_2)

CO_2 is mainly produced by heterofermentative LAB under anaerobic condition. The inhibitory effect of CO_2 acts to inactive the activity of decarboxylation enzymes as well as an altering in cell membrane permeability (Martin *et al*., 2003).

4.4 Diacetyl

Diacetyl is produced by all genera of LAB through citrate fermentation. It effectively inhibits Gram-negative bacteria by inactivating with arginine utilization (Jay, 1982). It has been reported that at 344 ng/ml of diacetyl could inhibit *Escherichia coli*, *Aeromonas, Listeria* and *Salmonella* (Ammor *et al*., 2006).

4.5 Low molecular weight antimicrobial substances

There are several reports on the low molecular weight components with antimicrobial activity by LAB.

4.5.1 Reuterin

Reuterin is the mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde (β-HPA). LAB produced reuterin at stationary phase culture under anaerobic condition. It shows broad-spectrum effect against Gram-positive and Gram-negative bacteria as well as molds, yeasts and protozoa (Cleusix *et al*., 2007; Montiel *et al*., 2014). Reuterin has been used as a bio-preservative agent in various meat and dairy products, and it effectively inhibited *Lis. monocytogenes* in cold-smoked salmon (Montiel *et al*., 2014). In addition, previous study reported that the reuterin in combination with nisin and lactoperoxidase showed the effective inhibition of *Lis. monocytogenes* and *S. aureus* in dairy product (Arqués *et al*., 2008).

4.5.2 Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, which can prohibit bacterial strain closely-related or non-related to produced bacteria, but will not harm the bacteria themselves by specific immunity proteins. Bacteriocins produced by LAB are mostly heat-stable, wide-range of pH stability, and are degradable by proteolytic enzymes. In addition, LAB bacteriocins are safe to be used as antimicrobial agent for further applications (Yang *et al*., 2014). For example, nisin A and nisin Z were produced by certain strains of *Lc. lactis*, their used as a bio-preservative agent in foods more than 50 countries (Gálvez *et al*., 2007; Campion *et al*., 2013; Gao *et al*., 2014; Perin and Nero, 2014). They are considered as safe antimicrobial agent due to their activity showed specifically antagonistic activity against the targeted bacteria at very low level (nano-molar level). However, the most

antibiotics must be at the micro-molar level for inhibitory activity. Furthermore, bacteriocin-producing LAB did not show side effects and resistance of bacteria in the practical use (Zendo, 2013). The characteristic of bacteriocins-producing LAB is proteinaceous nature to be degraded easily in the human body and in the environmental condition. The bacteriocins-producing LAB are varied types depending on species and strains of LAB. These compounds are classified into 3 main groups, Class I, Class II and Class III.

4.5.2.1 Classification of bacteriocins

The bacteriocins produced by bacteria including Gram-positive and Gram-negative bacteria have been divided into different classes based on the criteria such as producer strains, molecular weight, chemical structures, physical properties, mode of actions (Cotter *et al*., 2005). The classification of bacteriocins in this thesis mainly focused on bacteriocins-producing LAB are shown in Table 1.

Class I: Lantibiotics

Lantibiotics consist of small, post-translationally modified peptides which contained the intramolecular ring structures (Fig. 1). The peptide ring structure formed by modified thioether amino acids including lanthionine and β-methyllanthionine residues (Perin and Nero, 2014). Recently, over 40 lantibiotics have been characterized, and their physical and chemical properties found to be more diverse than what were originally thought, making it increasingly difficult to integrate a platform for subtyping the various lantibiotics. For example, two nisin variants (nisin A and nisin Z diverge only in one amino acid residue) have been discovered in *Lc. lactis*. Moreover, the novel nisin variant, nisin F, Q and U have been found in *Lc. lactis* subsp. *lactis* F10, *Lc. lactis* 61-14 and *Streptococcus uberis* 42 as well as showed the difference in amino acids 3%, 12% and 59%, respectively, when comparison with nisin A (Wirawan *et al*., 2006; Fukao *et al*., 2008; De Kwaadsteniet *et al*., 2008; Perin and Nero, 2014).

Class II bacteriocin: non-lanthionine

Class II bacteriocins are small molecules (<10 kDa), non-lanthionine as well as heat-stable peptides. They are unmodified amino acid residues at post-translation process with the exception of cysteine residues involving disulfide bond and oxidized-methionine (Himeno *et al*., 2012). The class II bacteriocins have comparatively narrow-spectrum antagonistic activity that showed inhibition of

Gram-positive bacteria, especially members of the genera *Enterococcus*, *Lactobacillus, Pediococcus* and *Listeria*. The class II bacteriocins divided into 4 subgroups (Cotter *et al*., 2005).

Subclass IIa bacteriocins are the largest subgroup that exhibited inhibitory activity against *Listeria*. These peptides have a consensus sequence of YGNGVXC within the N-terminal of the peptide chain (Fig. 2) (e.g., enterocin A) (Khan *et al*., 2010).

Subclass IIb are two-peptide bacteriocins which required both peptides for the best of inhibitory effect. The structural gene of the two active peptides are in the same operon (e.g., plantaricin EF) (Ekblad *et al*., 2016).

Subclass IIc bacteriocins are antimicrobially active circular peptides (e.g., lactocyclicin Q) (Sawa *et al*., 2009).

Subclass IId bacteriocins are leaderless bacteriocins (e.g., weissellicin Y) (Masuda *et al*., 2012).

Class III bacteriocins (large protein bacteriocins)

Class III bacteriocins are high molecular weight bacteriocins (>30 kDa) and heat-labile antibacterial proteins usually composed of different domains. Class III can be further subdivided into two distinct groups. Group A bacteriocins are the bacteriolytic enzymes which killing the sensitive strains by lysis of the cell well. For example, colicin is one of the well-characterized Class III bacteriocin. It commonly contains three domains, including receptor binding, translocation, and lethal domain. Previous study reported that zoocin A is one of the best-characterized LAB bacteriolysins (Gargis *et al*., 2009). It shows antagonistic activity against other streptococci by cleaving the peptidoglycan cross-links of the target cell wall.

Group B bacteriocins are non-lytic proteins which reveal their bactericidal effect without causing concurrence cell lysis. Dysgalacticin produced by *S. pyogenes* can be bind to the glucose and/or mannose phosphotransferase system (PTS), resulting in the inhibition of the sugar uptake, and also causes a membrane leakage of small molecules (Swe *et al*., 2009). On the other hand, caseicin from *Lb. casei* prohibits the DNA and proteins biosynthesis in target bacteria (Müller and Radler 1993).

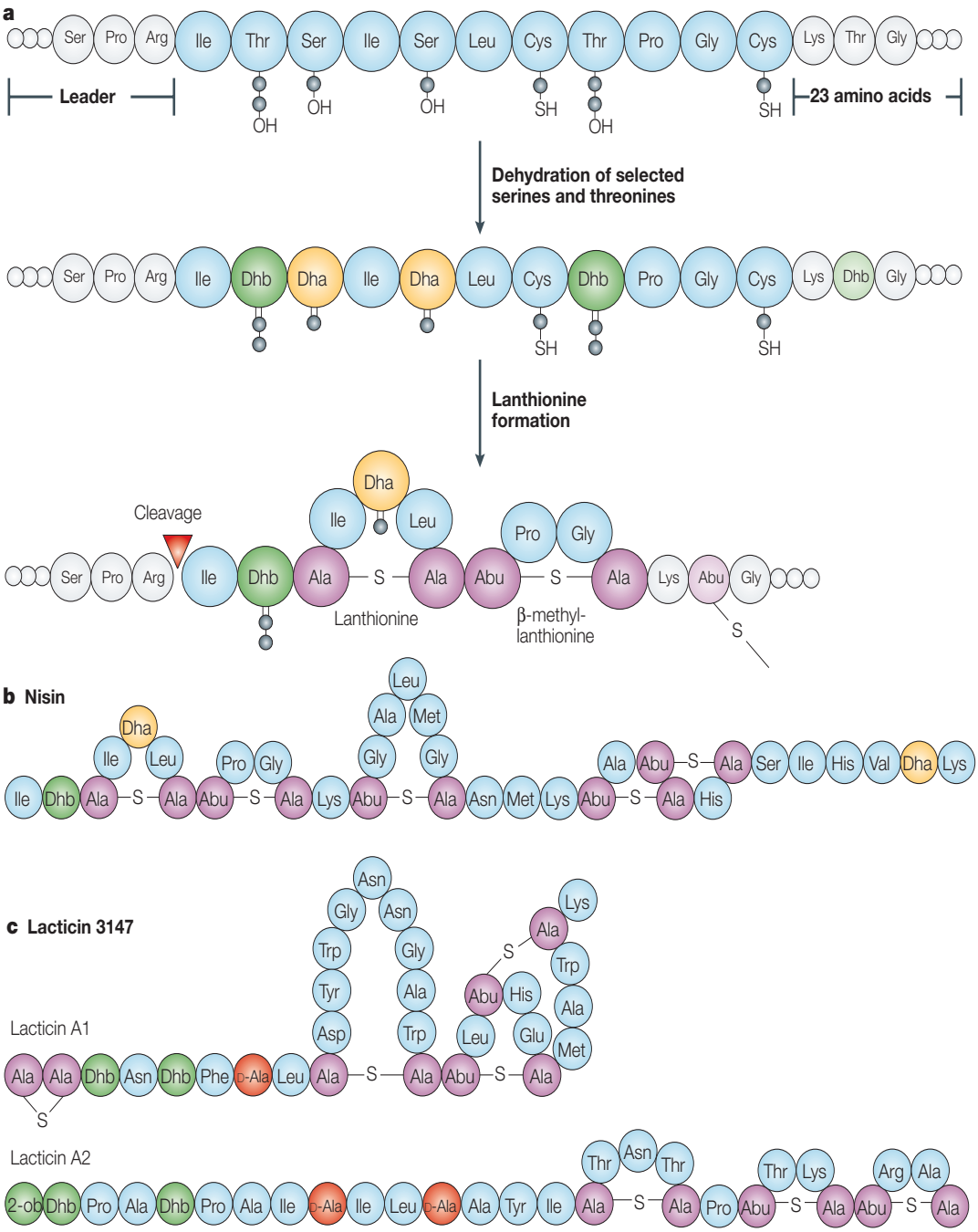

Figure 1 Lanthionine synthesis and lantibiotic structure. As shown in **a**): Lanthionine residues are formed when an enzymatically dehydrated serine (dehydroalanine, Dha) condenses with the sulphydryl group of a neighbouring cysteine (Cys). **b**): Nisin can be composed of a single peptide. **c**): two peptides acting in synergy (lacticin 3147). Source: Cotter *et al*. (2005)

Table 1 Bacteriocin substances produced by lactic acid bacteria (LAB).

Bacteriocin classification	Strains	Bacteriocins	References
Class I			
	Ent. faecalis NKR-4-1	Enterocin W	Sawa *et al*. (2012)
	Lc. lactis	Nisin A	Perin and Nero (2014)
		Nisin Z	Perin and Nero (2014)
		Nisin F	De Kwaadsteniet *et al*. (2008)
		Nisin Q	Fukao *et al*. (2008)
		Nisin U	Wirawan *et al*. (2006)
		Lacticin 481	García-Cayuela *et al*. (2017)
		Lacticin 3147	García-Cayuela *et al*. (2017)
Class II			
Class IIa	*Ent. faecium* NKR-5-3	Enterocin NKR-5-3C	Himeno *et al*. (2012)
	Ent. faecium	Enterocin A	Khan *et al*. (2010)
	Ent. mundtii	Munditicin	Zendo *et al*. (2006)
	Leu. pseudomesenteroides	Leucocin A	Sawa *et al*. (2010)
	P. pentosaceus	Pediocin PA-1	Garsa *et al*. (2014)
Class IIb	*Lc. lactis*	Lactococcin Q	Zendo *et al*. (2006)
	Ent. faecium	Enterocin X	Hu *et al*. (2010)
	Lb. plantarum	Plantaricin EF	Ekblad *et al*. (2016)
Class IIc	*Lactococcus* sp.	Lactocyclicin Q	Sawa *et al*. (2009)
	Leu. mesenteroides	Leucocyclicin Q	Masuda *et al*. (2011)
Class IId	*Lc. lactis*	Lacticin Q	Fujita *et al*. (2007)
		Lacticin Z	Iwatani *et al*. (2007)
	W. hellenica	Weissellicin M	Masuda *et al*. (2012)
		Weissellicin Y	Masuda *et al*. (2012)
Class III	*Lb. crispatus*	Helveticin M	Sun *et al*. (2018)

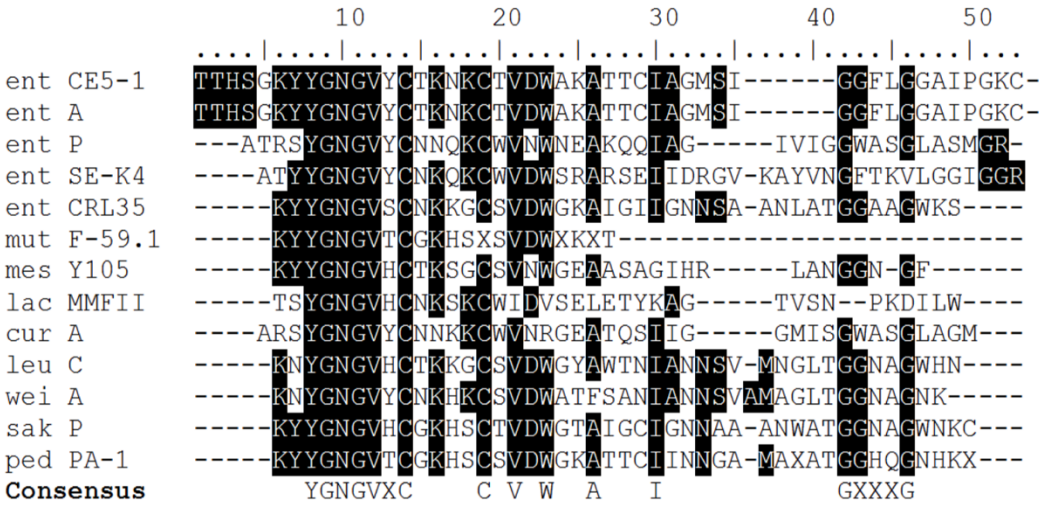

Figure 2 Alignment of the putative amino acid of Class IIa bacteriocin. (ent: enterocin; mut: mutacin; mes: mesenteriocin; lac: lactococcin; cur: curracin; leu: leucocin; wei: weissellin; sak: sakacin; ped: pediocin).

Source: Saelim *et al*. (2015)

5. Biosynthesis and regulation of bacteriocins

5.1 Bacteriocin biosynthesis

Recently, the scientific data show that genes encoding bacteriocin production are organized in operon clusters. Several researchers have been proposed that the genetic determinants of bacteriocin located on mobile genetic elements including bacterial chromosome (Kuipers *et al*., 1995; Chatterjee *et al*., 2005; Dimov *et al*., 2005). In generally, the bacteriocin production needs at least two genes: one structural peptide gene and another one that encodes an immunity protein for self-protection. Previous studies reported that some bacteriocin expression needs also a specific export machinery, as well as is subjected to some regulation factors, which make bacteriocin operons much more complex. Many publications have been described for lantibiotics operons are more complex than those encoding non-lantibiotics because they need additional genes encoding enzymes for post-translational modifications and translocation of bacteriocin across the membrane. (McAuliffe *et al*., 2001; Dimov *et al*., 2005).

The major pathways for Class I (lantibiotics) bacteriocins biosynthesis can be described by using the pathway followed by the well-known nisin, although there are slight differences for the non-lantibiotic bacteriocins as they do not need incorporation of unusual amino acids. The nisin biosynthesis of *Lc. lactis* strains is encoded by a cluster of 11 genes which is proposed to be transcriptionally arranged as *nisA(Z)BTCIP*, *nisRK*, and *nisFEG*, and is located on a large conjugative nisin-sucrose transposon (Fig. 3) (Kuipers *et al*., 1995; Kleerebezem and Quadri, 2001; Duhan *et al*., 2013). The structural gene of *nisA(Z)* gene is translated to nisin A(Z) precursor peptide consisting of 57-amino acid residues, including a 23-amino acid residues of leader peptide. The ribosomally synthesized of nisin is post-translational modified at the inner side of the membrane by an intracellular membrane-associated enzyme complex corresponding by *nisB* and *nisC* genes (Kleerebezem and Quadri, 2001; Chatterjee *et al*., 2005; Cheigh and Pyun, 2005). The amino acid residues including serine and threonine residues are dehydrated to become dehydroalanine and dehydrobutyrine.

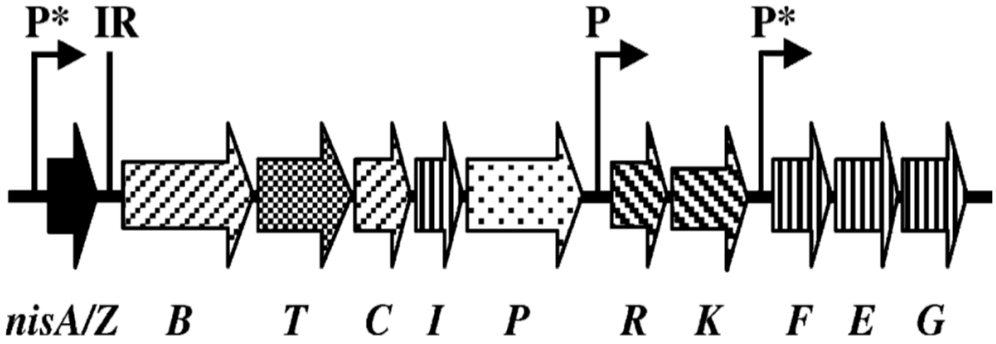

Figure 3 Organization of nisin biosynthetic gene clusters.
Source: Cheigh and Pyun (2005)

Subsequently, the dehydrated of amino acid residues are coupled to cysteines residues to be formed a thioether bonds that produced the lantionine and β-methyllantionine rings in the nisin peptide (Kuipers *et al*., 1995). Then, the putative transporter protein of ABC translocator is encoded by *nisT* gene that translocated the fully modified nisin precursor across the cytoplasmic membrane. In the last step of nisin biosynthesis, the leader peptide is cleaved by the cell-wall-anchored serine protease (NisP) involving *nisP* gene to release a nisin active form (Kleerebezem and Quadri, 2001; McAuliffe *et al*., 2001; Chatterjee *et al*., 2005; Cheigh and Pyun, 2005).

Most of the genes encoding of Class II bacteriocins biosynthesis may be either plasmidial localization or chromosomal localization (Ennahar *et al*., 2000; Ditu *et al*., 2014). Several authors reported that sakacin P, carnobacteriocins B2 and BM1 and plantaricin ST31 (Huhne *et al*., 1996; Quadri *et al*., 1997; Ennahar *et al*., 2000) are chromosomally encoded, whereas plantaricin 423 and pediocine PA-1 are plasmid encoded (Miller *et al*., 2005; Todorov, 2009). The class II bacteriocins production are organized within operon clusters and normally consist of a structural gene encoding the prepeptide, and dedicated immunity gene, an ABC-transporter gene for translocation through the membrane, as well as an accessory protein gene for exporting the bacteriocin (Ennahar *et al*., 2000; Ditu *et al*., 2014; Mokoena, 2017). Most class II bacteriocins are synthesized as an inactive prepeptide form carrying a N-terminal leader peptide and a distinctive double-glycine proteolytic processing site. Then, the leader sequence is cleaved from the active site represented by two-glycine residues, generating the active form of bacteriocin molecule. Afterwards, ABC transporter and its accessory protein transferred the active bacteriocin across cell membrane (Ennahar *et al*., 2000; Ditu *et al*., 2014).

Moreover, the bacteriocin-producing strains had a system for protection themselves from the toxic effect of bacteriocin inside the cell by producing specific immunity proteins. The immunity gene and the structural bacteriocin gene are most likely located on the same operon. Several studies reported that LAB had both the *LanI* and the *LanEFG* systems for self-protection. LanI protein interacts with bacteriocin at the surface of cytoplasmic membrane and by blocking bacteriocin to penetrate into the cell membrane for preventing pore formation (McAuliffe *et al*., 2001; Stein *et al*., 2003) (Fig. 4). Additionally, LanFEG protein was control the quantity of baceriocin molecule by transporting it from the cytoplasmic membrane into extracellular space before/during pore formation (McAuliffe *et al*., 2001; Stein *et al*., 2003; Mokoena, 2017).

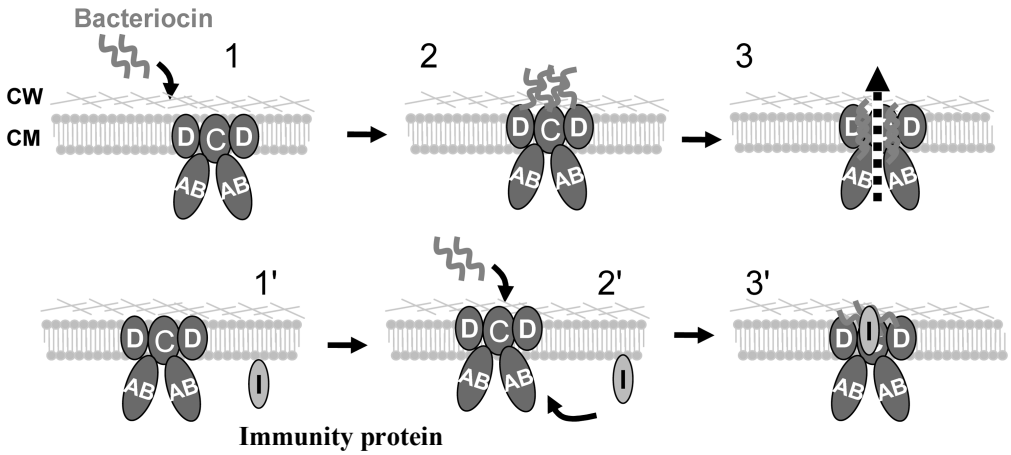

Figure 4 Model for the mechanism on one-peptide class II bacteriocin interact with their receptor and permeabilize bacterial membranes (1-3), and on how the bacteriocin immunity interacts with the receptor to block the permeabilizing effect of the bacteriocin (1′-3′). The receptor mannose-PTS complex comprises the proteins AB, C, and D. the immunity protein (I) is shown in the figure.
Source: Nes *et al*. (2007)

5.2 Regulation of bacteriocins expression

Expressions of bacteriocin genes are usually subjected to regulation by external induction factors (IF). Bacteriocin-producing LAB is controlled by a "quorum-sensing" mechanism. The quorum-sensing system controls an important biological process in bacteria such as natural genetic transformation, virulence and sporulation. In Gram-positive bacteria, quorum sensing is predominantly mediated by peptide pheromone. The IF is believed to bind specifically to the correspondent histidine protein kinase and to activate it to phosphorylate the response regulator, which then stimulates transcription of the target genes, most probably by binding to specific imperfect direct repeats found in many bacteriocin genes cluster (Fig. 5) (Dimov *et al*., 2005). IF for a given bacteriocin operon can be produced either by the bacteriocins-producing strain or by other strain belonging to the same or other species or genera. When the inducer peptide is generated by the bacteriocin producer itself, there is an autoregulation of bacteriocin biosynthesis. The IF can be a dedicated peptide encoded by a respective gene or the bacteriocin molecule itself.

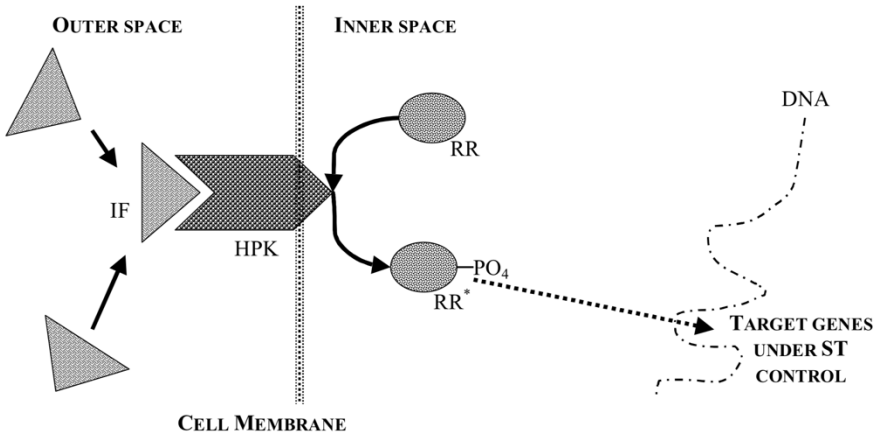

Figure 5 Three component signal transduction pathways used by many bacteriocin operons in quorum-sensing regulation. Abbreviations: *IF* induction factor; *HPK* histidine kinase; *RR* and *RR** response regulators

Source: Dimov *et al*. (2005)

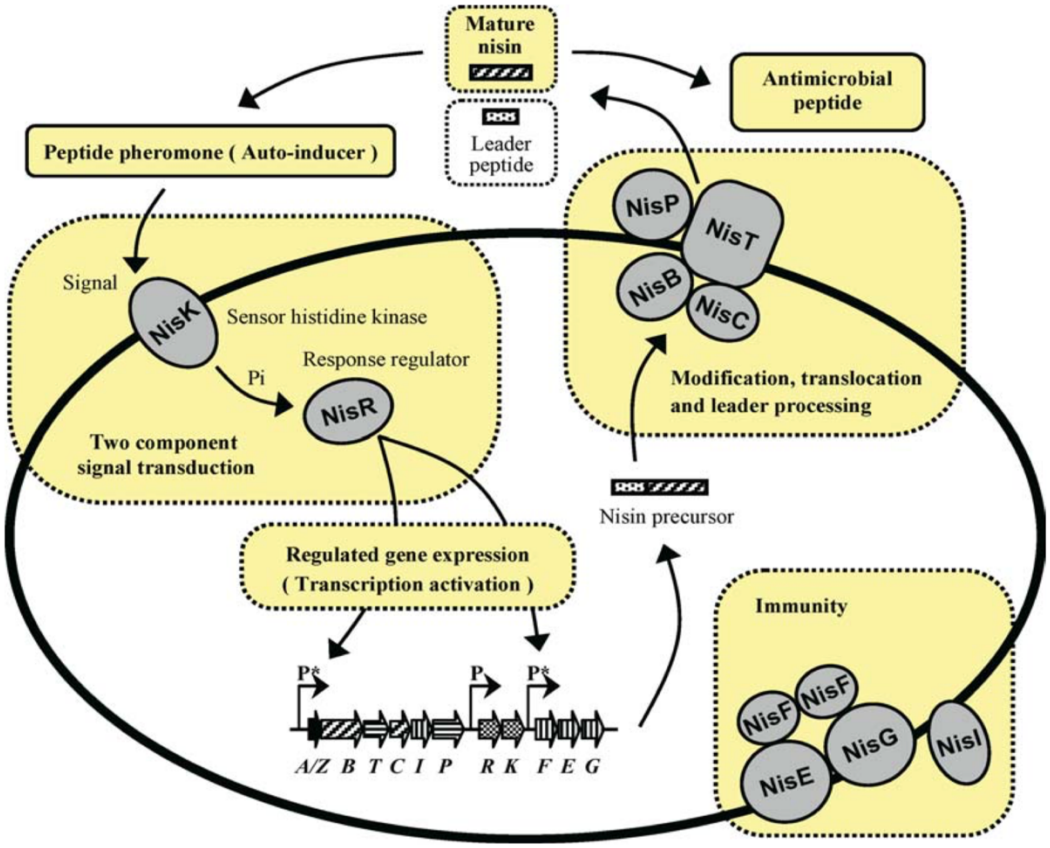

Figure 6 Schematic representation of nisin biosynthesis and regulation in *Lc. lactis*.

Source: Cheigh and Pyun (2005)

For example, nisin molecule interacts with a sensor histidine kinase produced by the gene *nisK* and disposed on the cell membrane. The histidine kinase phosphorylates the response regulator product of the gene *nisR*, followed by binding to the imperfect direct repeats found in the promoter regions. This event leads to transcription activation of the nisin gene cluster and finally to the secretion of the more nisin molecules, thus closing the cycle of auto activation (Kuipers *et al*., 1995; Kleerebezem and Quadri, 2001; Cheigh and Pyun, 2005) (Fig. 6).

6. Mechanism of action of bacteriocins-producing LAB

The action of bacteriocin produced by LAB inhibited a closely related LAB spices or other Gram-positive species. The bacteriocin-producing LAB such as nisin is antagonistic activity against a broad variety of bacteria including strains of bacilli, lactobacilli, lactococci and staphylococci (de Arauz *et al*., 2009). The lantibiotic bacteriocins including nisin, subtilin and lacticin 3147 act to disrupt the proton motive force (PMF), inhibit transport of amino acids and lead to release of intracellular content (H^+, Mg^{2+}, NaCl, amino acids and other small molecules in cytosol) resulting in cell death (Breukink *et al*., 1999; Wiedemann *et al*., 2001; Cotter *et al*., 2005). Previous studies have been described for the pore-formation by nisin to insert into lipid bilayers through ionic interactions with the phospholipid head groups (Garcerá *et al*., 1993; Driessen *et al*., 1995; McAuliffe *et al*., 2001) (Fig 7). The positively charged nisin molecules bind the negatively charged carboxyfluorescein molecules as well as cross the membrane as a nisin-anion complex. This activity is strongly inhibited in liposomes composed of anionic phospholipids result in the pores forming on cell membrane (Driessen *et al*., 1995; McAuliffe *et al*., 2001). Other mechanism of action well recognized and reported by different authors, showed that nisin uses Lipid II as a ‘docking molecule’ to initiate a process of membrane insertion and pore formation in a targeted cell (Breukink *et al*., 1999; Cotter *et al*., 2005). The second mode of action of nisin is specific recognition and binding lipid II to form pores in target cell membrane (Fig. 8). Moreover, the binding of nisin to lipid II is displaced afterwards from its location in Gram-positive bacteria, leading to disrupts cell wall biosynthesis during cell division and bacteria are killed (Hasper *et al*., 2006; Christ *et al*., 2007).

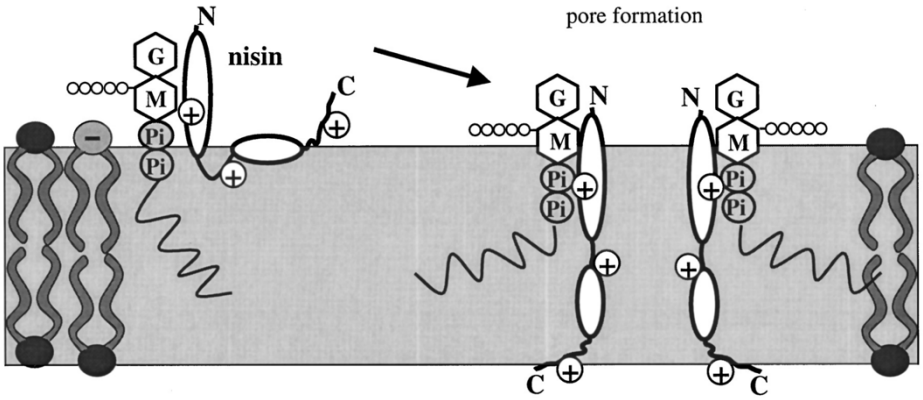

Figure 7 A schematic representation of the mode of action of nisin.

Source: modified from Wiedemann *et al*. (2001)

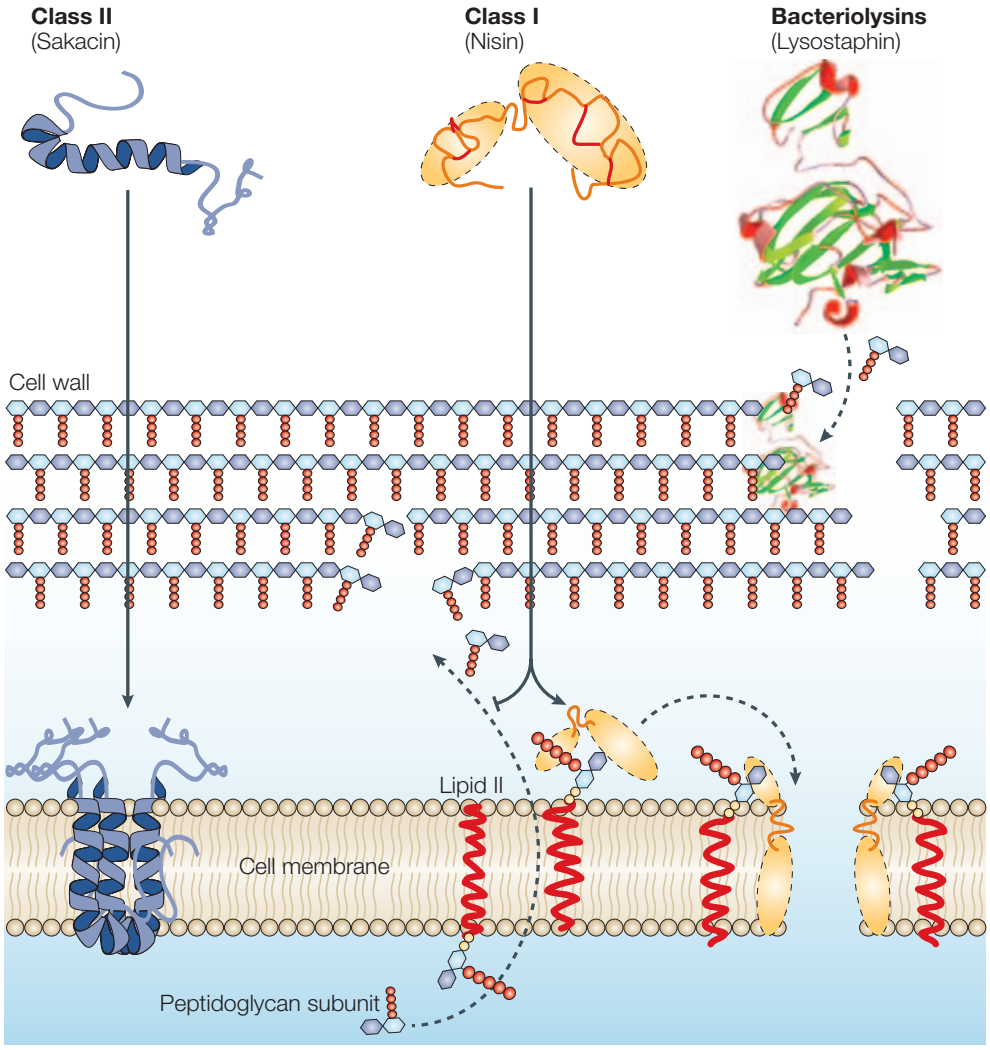

Figure 8 Mode of action of LAB bacteriocins.

Source: Cotter *et al*. (2005)

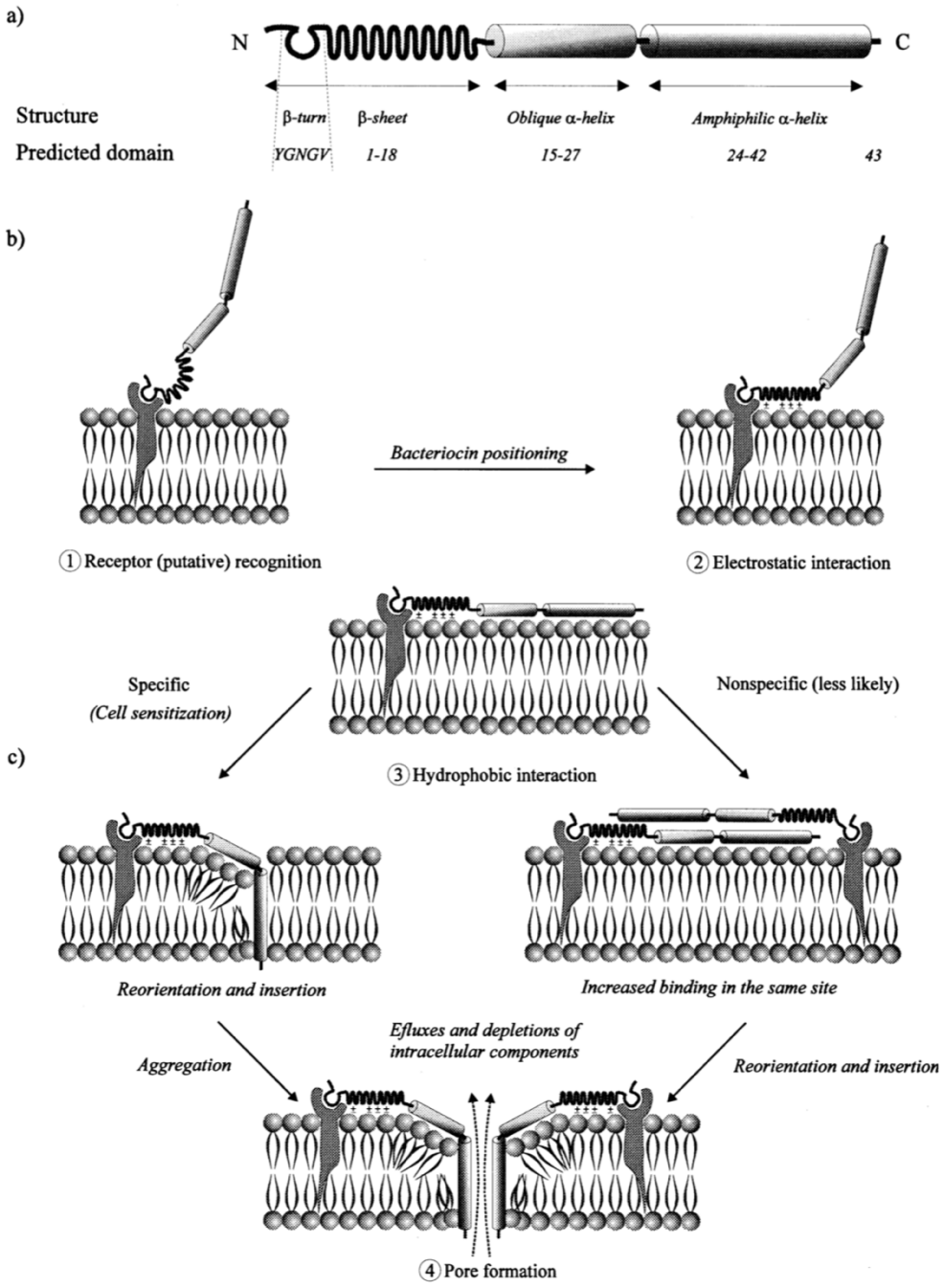

Figure 9 Schematic representation of the structure of a model class-IIa bacteriocin and the predicted location its domains with respect to target cell membrane: (a) bacteriocin predicted structural domains; (b) possible interactions of each domain with the membrane surface; (c) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and hydrophilic face is shaded light.
Source: Ennahar *et al*. (2000)

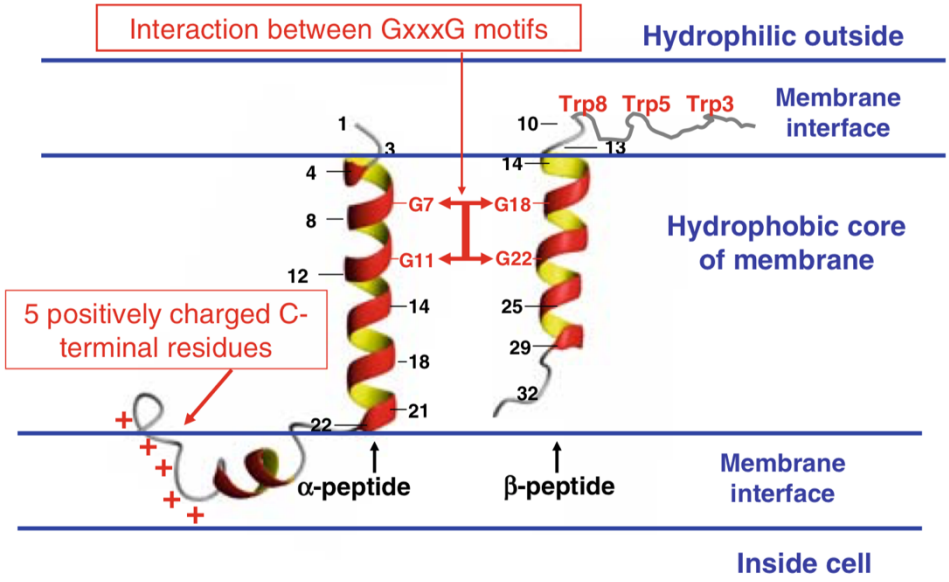

Figure 10 The structural model of lactococcin G and its orientation in target-cell membranes.

Source: Nissen-Meyer *et al*. (2010)

The mode of action of class IIa bacteriocins such as pediocin PA-1 is leading to translocation of the peptides across the lipid bilayer. Previous study reported that pediocin PA-1 could bind to cytoplasmic membrane, insertion of bacteriocin molecules in the membranes and formation of the poration complex (Ennahar *et al*., 2000). This process leads to cell death because of the low intracellular ATP level and the inability to carry out active transport of nutrients and to maintain sufficient concentrations of cofactors, such as K^+ and Mg^{2+} (Fig. 9). Additionally, mechanism inhibition of two-peptide bacteriocins (class IIb bacteriocins) on sensitive bacteria is similar to the mode of action of the pediocin-like. Several researches have been proposed that two-peptide bacteriocins form a membrane-penetrating helix-helix structure interacting with an integrated membrane protein, thereby activating membrane-leakage as well as cell death (Oppegård *et al*., 2007; Nissen-Meyer *et al*., 2010) (Fig. 10). However, bacteriocins produced by LAB including lantibiotic and non-lantibiotic bacteriocins did not inhibit Gram-negative bacteria due to lipopolysaccharidic (LPS) composition of its outer layer which acts as a barrier to the action of the nisin on the cytoplasmic wall (Cao-Hoang *et al*., 2008; Gyawali and Ibrahim, 2014).

7. Screening and identification of bacteriocin-producing LAB.

Bacteriocin-producing LAB strains were isolated from various sources, including meat, dairy products, fermented sausages as well as fermented seafood. The first step in bacteriocin determination evaluated the inhibitory activity of the obtained bacteria isolated by microbiological testing. The spot-on-lawn method, agar well diffusion assay, disc diffusion method as well as microtiter plate assay are the most frequently detected in the bacteriocin activity (Kaškonienė *et al*., 2017). All methods are based on the inhibition growth of target strain to react the supernatant containing bacteriocin. Nevertheless, the inhibitory activity of bacteriocin depended on the appropriate media for the bacteria growth and indicator strain for antibacterial testing (Kaškonienė *et al*., 2017). After the positive bacteriocin activity testing, the identification of bacteriocin-producing LAB is performed by sugar fermentation pattern and 16S rDNA sequence by PCR technique (Biscola *et al*., 2013; Koral and Tuncer, 2014). The effect of proteolytic enzymes, pH and temperature on bacteriocin activity are performed in order to evaluate the characteristic of bacteriocins for the further application in food products. Several researchers reported that the bacteriocins were inactivated with different proteolytic enzymes, whereas, their remained inhibitory activity at wide-range of pHs and high temperature condition (Saelim *et al*. 2015; Hernández-Saldaña *et al*. 2016). In addition, the effect of NaCl content on antibacterial activity is very valuable data to be applied in different food matrices (Castro *et al*., 2011).

Recently, the information involving the bacteriocins-producing bacteria as well as their genomic data increases are very useful to apply the bioinformatic data for identification of bacteriocins or their producing strains (de Jong *et al*., 2010; Morton *et al*., 2015). Researchers have been developed the tools for identification of bacteriocins such as BACTIBASE (Hammami *et al*., 2007), BAGEL (de Jong *et al*., 2010; van Heel *et al*., 2013) and BOA (Morton *et al*., 2015) for reducing time-consuming antimicrobial testing. Moreover, other approach for screening bacteriocins-producing LAB based on their antimicrobial spectra and molecular masses was proposed by Zendo *et al*. (2008) and Zendo (2013) (Fig. 11). This system performed by molecular mass analyses using liquid chromatography/mass spectrometry (ESI-LC/MS) in combination with principal component analyses (PCA) of the antibacterial

activity spectrum of bacteriocin obtained from LAB isolates. It was found that using this system was able to rapidly identify novel bacteriocin-producing LAB strains and discard those that produce known bacteriocins, thus accelerating the discovery of novel bacteriocins including variants of those already reported (Zendo, 2013).

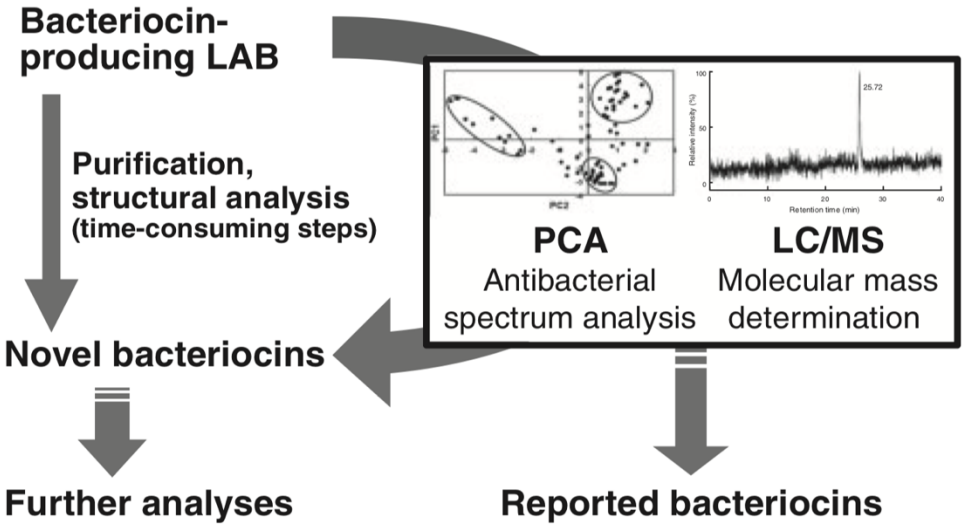

Figure 11 Scheme of the rapid screening system for novel lab bacteriocins.
Source: Zendo (2013)

8. Purification of bacteriocins

The bacteriocin-producing LAB did not use as preservative agents for commercial products because the high quality of commercial bacteriocin is the high cost of its production, of which the two major contributors are: (1) the cost of synthetic medium and nutritional supplements used for the growth of bacteriocin-producing cells and (2) the efficient separation processing of bacteriocins had an expensive technique for recovery of bacteriocins from the complex crude feedstock (Bali *et al*., 2016; Jamaluddin *et al*., 2017; Kaškonienė *et al*., 2017). The conventional purification methods of bacteriocins have been reported in previous studies, such as ammonium sulfate precipitation, pH-mediated cell adsorption/desorption, membrane filtration and chromatographic methods (Cheikhyoussef *et al*., 2009; Jamaluddin *et al*., 2017; Kaškonienė *et al*., 2017). Several studies reported that the most popular purification method is the use of ammonium sulfate precipitation and Sepharose-based ion exchange chromatography (Table 2) (Hata *et al*., 2010; Gao *et al*., 2015; Kumar *et al*.,

2015; O'Connor *et al*., 2015; Saelim *et al*., 2015). The rapid purification methods have been reduced to two or three-steps procedure for purification of bacteriocins. Batdorj *et al*. (2006) who studied two-peptides bacteriocin from *E. durans* that was successfully purified by three-steps procedure including cation exchange, reverse-phase column R1 and RP-HPLC. Two peptides obtained from RP-HPLC represented enterocin A511A and enterocin A511B, which showed purification factors of about 1650 and 2250-fold. Additionally, the two-steps purification (ammonium sulfate precipitation and gel exclusion chromatography) of bacteriocin produced by *B. subtilis* R75 has been reported by Sharma *et al*., (2011), resulting in 22.3 purification fold and 22.1% yield. However, these techniques are more cost-effective to production of high quality bacteriocins for commercial markets. In addition, several studies reported that the novel approach for bacteriocins purification base on the chromatography methods including aqueous two-phase systems (ATPS), and aqueous micellar two-phase systems (AMTPS) (Lappe *et al*., 2012; Jozala *et al*., 2013; Jamaluddin *et al*., 2017). These techniques were developed for reducing the time consuming, cost-effective and laborious. The ATPS is a purification technique by formation two phases with two-incompatible polymers or polymer/inorganic salts. These compounds were mixed in water above a certain critical concentration (Lappe *et al*., 2012). Lappe *et al*. (2012) purified cerein 8A using ATPS technique and obtained a higher yield (87%) comparing with the conventional method. Abbasiliasi *et al*. (2014) successfully purified the bacteriocin produced by *P. acidilactici* Kp10 using an ATPS method that comprised of 26.5% polyethylene glycol (PEG, 8000) and 11% of sodium citrate, showing a yield 81.8% and 8.4 purification fold. Furthermore, an AMTPS technique separated a desired biomolecule using a non-ionic surfactant for formation of binary phases. This method has been proposed by Jozala *et al*. (2013) for purification of nisin that showed a greater activity than conventional purification method. Although, the alternative methods demonstrated high performance comparison with conventional counterparts (in term of activity yield), they gave a lower degree of purification (Lappe *et al*., 2012; Jamaluddin *et al*., 2017).

Table 2 Purification possibilities for bacteriocins.

Techniques	Material used	Bacteriocin
Precipitation	Ammonium sulphate	Plantaricin ASM1 (Hata *et al*., 2010); enterocin (Saelim *et al*., 2015); nisin Z (Hwanhlem *et al*., 2013)
Ion exchange chromatography	Sepharose-based, i.e. octyl-, sulphopropyl-, phenyl-, carboxymethyl	Enterocin (Zendo *et al*., 2005); plantaricin ASM1 (Hata *et al*., 2010); enterocin LR/6 (Kumar *et al*., 2015); garviecin LG34 (Gao *et al*., 2015); nisin H (O'Connor *et al*., 2015)
	Toyopearl CM-650M OASIS-HLB column	Enterocin ON-157 (Ohmomo *et al*., 2000) Thermophilin 1277 (Kabuki *et al*., 2007)
	HiTrap SP cation exchange column	Nisin Z (Hwanhlem *et al*., 2013)
Absorption	Amberlite XAD-16	Sakacin D98a, sakacin D98b and sakacin D98c (Sawa *et al*., 2013)
Solvent extraction	Diethyl ether, Isopropanol, Methanol/chloroform Acetate buffer with NaCl and methanol	Acidocin CH5 (Chumchalová *et al*., 2004) Lactococcin BZ (Şahingil *et al*., 2011) Nisin (Sadiq *et al*., 2014) Nisin A and nisin Z (Ko *et al*., 2015)

9. Application of bacteriocins in food products

Nowadays, the consumers are particularly conscious of the health concerns relating with food additives. The chemical additives including antibiotics have been used to inhibit the spoilage or pathogenic bacteria in food matrices. The releasing of antibiotics could enter the agricultural as well as foods to be induced the antibiotic resistant genes in bacteria. If human pathogenic bacteria acquired the antibiotic resistance genes could become resistant to antibiotics, and a potential risk to human health. In contrast, bacteriocins have effectiveness against food spoilage and pathogenic bacteria and also due to its proteinaceous nature which made it safer for human consumption. It is assumed to be rapidly degraded by proteolytic enzymes in gastrointestinal track (Cleveland *et al*., 2001). Bacteriocins as bio-preservatives agent are a Generally Recognized As Safe" (GRAS) status (Cotter *et al*., 2005). Several food scientists used LAB bacteriocins for controlling spoilage and pathogenic bacteria growth in both fermented and non-fermented foods by using broad- and narrow-host-

range bacteriocins (Settanni and Corsetti, 2008; Sobrino-López and Martín-Belloso, 2008; Lauková *et al*., 2010a). For example, nisin has been approved for utilization as a preservative in many kinds of foods by the US Food and Drug Administration (FDA, 1988), commercially available as Nisaplin® (Danisco, Grindsted, Denmark). Another commercially bacteriocin is pediocin PA-1 produced by *P. acidilactici* and marketed as ALTA® 2431 (Kerry Bioscience, Carrigaline, Ireland). Regarding bacteriocins application should not be used as the primary processing step or barrier to prevent microbial growth, but rather they can be combined with the hurdle technologies to decrease the possibility of food-borne disease (Deegan *et al*., 2006).

9.1 LAB bacteriocins for improvements in food safety

The European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported numbers of food-borne illnesses and intoxications are still on the increase since 2008. According to EFSA and ECDC reports demonstrated that human illness occurred from the food-borne pathogenic bacteria contaminants such as *Campylobacter* sp., *E. coli* (STEC), *Lis. monocytogenes, S. aureus* and *Salmonella* sp. (EFSA, 2017).

Biopreservation is a powerful and natural tool to extend shelf life and to increase the food safety by applying naturally occurring microorganisms and/or their inherent antimicrobial substances of defined quality and at certain quantities (Ghanbari *et al*., 2013). Several studies have been successfully used LAB bacteriocins by adding bacteriocin-producing cultures or crude extract bacteriocins for controlling pathogens and spoilage bacteria in foods (Benkerroum *et al*., 2003; Randazzo *et al*., 2009; Mitra *et al*., 2010; Oliveira *et al*., 2015). Several researches have been successfully reduced the spoilage and food-borne pathogenic bacteria on fresh lettuces after treating bacteriocins-producing LAB (Randazzo *et al*., 2009; Oliveira *et al*., 2015). From literatures revealed that LAB bacteriocins could be applied in the food products as naturally food preservatives. Its bacteriocins are safe for human consumption as well as have a potential of inhibition of spoilage and food-borne pathogenic bacteria. Additionally, bacteriocins-producing LAB have the ability of BAs reduction as toxicological effect on human. Therefore, LAB bacteriocins are useful to apply into food stuffs for enhancing safety and prolong shelf- life.

9.2 LAB bacteriocins for enhancing safety in meat products

For improving safety of fresh meat and fermented meat products by application of bacteriocin-producing *Lb. sakei* CECT 4808 and *Lb. curvatus* CECT 904^T as bioprotective culture on slices beef. Samples inoculated with the *Lb. sakei* strain and the combination of the two strains (*Lb. sakei and Lb. curvatus*) had significantly ($p<0.05$) lower spoilage microbial counts than those inoculated with the *Lb. curvatus* strain alone or the controls, while both chemical parameters (including lipid oxidation and abnormal odor scores) were also significantly ($p<0.05$) improved by the former (Katikou *et al*., 2005). Zhang *et al*. (2010) inoculated bacteriocin-producing *Lb. pentosus* 31-1 into fresh pork and found that it could inhibit the growth of microflora, especially *Listeria* and *Pseudomonas* during chilled storage. The sample was treated by pentocin 31-1 (at final concentration 80 AU/ml) could extend the shelf life to 15 days and showed good sensory characteristics. Di Gioia *et al*. (2016) showed the possibility of using *Lactobacillus* spp. strains as protective culture in pork ground meat for fermented salami preparation. They found that *Lb. plantarum* PCS20 was able to survive and show the inhibitory activity against *Clostridium* strains in ground pork. Furthermore, de Souza Barbosa *et al*. (2015) found that partially purified sakacin from *Lb. curvatus* as bio-preservative agent could be reduced a 2 log CFU/g of *Lis. monocytogenes* at the end of 30 days of salami maturation when comparing with the control. This result was in agreement with study of Du *et al*. (2017) who applied the two partially purified enterocin from *E. duran* 152 as an anti-listeria agent in deli-ham. The obtained result exhibited that at 400 AU/ml of bacteriocins could inhibit listeria growth in deli-ham for at least 10 weeks at 8°C, comparing with nisin as control treatment.

9.3 LAB bacteriocins for enhancing safety in seafood products

Recently, the several researches interest the using of LAB protective culture and/or their bacteriocins in fish and seafood products to control microbial deterioration without negative impact on the sensory quality of the product. As previous study of Anacarso *et al*. (2014) who applied *Lb. pentosus* 39 or their bacteriocin to be used as protective culture or bio-preservative agent in fresh salmon fillets for prolong shelf-life. The sample was inoculated with *Lb. pentosus* 39 and its bacteriocin that

significantly reduced a 2.8 log CFU/g for *Aeromonas hydrophila* and 5.8 log CFU/g for *Lis. monocytogenes* counts, respectively, comparing with control under simulated cold-chain break conditions. However, the sample treated with its bacteriocin alone showed a less inhibitory activity than combining of bacteriocin and culture cells. Schelegueda *et al*. (2015) studied the inhibitory effect of bacteriocin-producing *E. mundtii* regarding its potential use for fish preservation. The results presented that the combination of bacteriocins with sodium lactate and chitosan exerted a synergistic action on the inhibition the growth of *Lis. innocua*, *Shewanella putrefaciens* as well as the psychrophilic flora isolated from fish. The use of mention mixtures would be useful to decrease the amount of antibiotics added into foods and to prevent the growth of spoilage and pathogenic bacteria. Lv *et al*. (2018) found that the novel bacteriocin produced by *Lb. plantarum* DY4-2 exhibited a bactericidal effect against fish pathogenic and spoilage bacteria, such as *A. sobria*, *Lis*. *monocytogenes*, *Pseudomonas fluorescens*, *P. aeruginosa* and *V. parahaemolyticus*. The fish fillets were treated by partially purified bacteriocin DY4-2 to be reduced by a 2.7 log CFU/g of *P. fluorescens* at 4 °C storage for 12 days. Moreover, a significant decrease in total volatile base nitrogen and total viable counts was observed in bacteriocin DY4-2 treated samples. The obtained result demonstrated that bacteriocin DY4-2 had potential to be used as seafood bio-preservative for improving safety and prolong shelf-life.

Furthermore, several researchers applied the bacteriocin-producing strain to be used as starter culture or protective culture for reduction of BAs accumulation in fermented seafood products. As previous study reported that bacteriocin produced by *Ent. faecalis* A-48-32 can be reduced BAs content in sardines (especially, histamine and tyramine) and had the highest antagonistic activity against histamine- and tyramine-producing LAB. Besides, the enterocin A-48-32 was used as bio-preservative agent in sardines and found that it decreased the level of histamine and tyramine approximately 6 and 2 times with respect to untreated control during storage in modified atmosphere (Ananou *et al*., 2014).

9.4 Antimicrobial packaging for increasing food safety

The antimicrobial biodegradable-packaging is interest of several researchers because resolving an environmental problem by reduction a solid waste, polluted groundwater as well as air pollution. Film-bacteriocin packaging can be inhibited the undesirable microorganisms for improving food safety and prolong shelf-life during storage and distribution (Massani *et al*., 2013; Guo *et al*., 2014). As previous study of Guo *et al*. (2014) who found that antibacterial films coating presented inactivation of *Lis*. *innocua* on ready-to-eat turkey meat. The various of LAB bacteriocins/biopolymers film-coating system on inhibition of undesirable growth have been studied, for example, nisin/chitosan (486/1.94 mg/cm^2) coating ratio showed significantly reduce a 5.0 log CFU/ cm^2 and 3.0 log CFU/cm^2 of *Lis. innocua* as well as *Sal.* Typhimurium, respectively. Woraprayote *et al*. (2013) developed an antibacterial biocomposite film system of polylactic acid (PLA)/sawdust particle (SP). The PLA/SP film coated with pediocin PA-1/AcH (11.6 μg protein/cm^2) effectively inhibited *Lis. monocytogenes* growth both *in vitro* and *in situ* condition (a raw sliced pork during the chilled storage). Massani *et al*. (2014) reported that gluten-based films cooperated with lactocin 705 and lactocin AL705 effectively inhibited *Lis. monocytogenes* in the artificially contaminated Vienna sausage during 45 days of chilled storage.

Moreover, researchers attempt to increase the inhibitory activity effectiveness of meat packing and other food grade antimicrobials to incorporate into film for synergistic action with bacteriocin. Pattanayaiying *et al*. (2015) found that pullulan film incorporated by nisin Z and lauric arginate (LAE) showed antagonistic activity against both Gram-positive and Gram-negative food-borne pathogens including *E. coli*, *Lis. monocytogenes*, *S. aureus* and *Salmonella* spp. *in vitro*. The active film containing a nisin Z with LAE reduced *Lis. monocytogenes* and *S. aureus* by 5.6 and 5.5 log CFU/cm^2 onto ham surfaces, respectively during chilled storage.

9.5 Requirement properties of bacteriocin-producing LAB used as protective culture or/and starter culture

Bacteriocin-producing LAB are widely used as bio-protective culture or/and starter culture in food product for improving safety and prolong shelf-life (Sarika *et al*., 2012; Zhang *et al*., 2013). Most LAB are considered as GRAS and QPS status by European Food Safety Authority (EFSA, 2007; EFSA, 2011b). However, alive microbial antagonist must be checked the safety aspects before taking into the accounts. The safety aspects of bacteriocin-producing LAB used as starter culture or protective culture for meat, seafood and fermented products did not exhibit any problematic antibiotics resistance (EFSA, 2012) nor BAs formation (Ammor and Mayo, 2007). Additionally, the alive cells or their metabolites did not affect on chemical, physical and sensory quality parameters of target products (Castellano *et al*., 2010).

9.5.1 Safety assessment of bacteriocin-producing LAB

9.5.1.1 Antibiotics susceptibility

Antibiotic resistance shows an increasing world-health threat that relate all major pathogens and antibiotic drugs. As previous study reported that the infection with resistance microbial complicated the treatment of diseases. The morbidity and mortality associated with infectious diseases were a significant increase (Levy and Bonnie, 2004). The antibiotic resistance characteristic is the major safety issues for starter culture selection because the antibiotic resistance determinants from LAB reservoir strains can transfer to bacteria in fermented foods. Consequently, these determinants can spread to microflora of human gastrointestinal tract as well as to pathogenic bacteria (Toomey *et al*., 2010). Several studies reported that antibiotic resistance profiles were distinction between intrinsic and acquired antibiotic resistances (Mathur and Singh, 2005; Hummel *et al*., 2007; Perin *et al*., 2014). An intrinsic resistance is inherent to a bacterial species or genus (naturally resistance) involving of the ability defense in microbial cell. These defend mechanisms related to inactivation of drug by bacterial enzyme and inaccessibility of the drug into the bacterial cell by decreasing drug uptake (Kumar and Schweizer, 2005). The intrinsic determinant is a minimal potential for horizontal transfer between bacterial species as well as poses no risk in non-pathogenic bacteria. Conversely, acquired resistance can be horizontally transferable of resistance genes among natural microbial communities (over species

and genus border) by conjugative transposon and the possession of integrons and insertion of mobile elements (like plasmids) (Huddleston, 2014). Several studies reported that genes of resistance to erythromycin, tetracycline and vancomycin have been found in *Enterococcus*, *Lactococcus*, *Lactobacillus* species isolated from fermented products (Mathur and Singh, 2005; Hummel *et al*., 2007). The antibiotics susceptibility of the microbial feed additive is considered by EFSA to QPS status (EFSA, 2018). The phenotypic of antibiotics susceptibility testing are determined using internationally standardized methods. The minimum inhibitory concentration (MIC) of the antibiotic has been suggested along with breakpoints classifying bacterial species (Table 3). Accordingly, the breakpoint cut-off values established by EFSA (2018) have been provided to distinguish strains with acquired resistance from susceptible strains.

The genotyping assays to identify resistant bacterial strains by PCR-based techniques have been used. Several researchers have been frequently found antibiotic resistant genes in LAB including tetracycline (*tetM*), erythromycin (*ermAM*), chloramphenicol (*cat*), streptomycin (*str*), streptogramin (*sat*) (Mathur and Singh, 2005; Ammor *et al*., 2008; Fraqueza, 2015). The genotyping result was clearly agreement with the phenotypic resistance profile (Ammor *et al*., 2008; Fraqueza, 2015). Nevertheless, a phenotypically resistant strain may be genotypically "susceptible" due to the fact that appropriate genes are not included in the test patterns, or that there are unknown resistance genes (Fraqueza, 2015). Therefore, the drugs resistance profile of LAB strains should be taken before use as starter culture in food products for preventing the resistance genes transferable to pathogens through food chain.

9.5.1.2 Biogenic amines formation

Biogenic amines (BAs) are generally considered as hazard compound in foods that derived by decarboxylation of corresponding free amino acids. They can be formed as a result of amino acid decarboxylase activity in spoilage and other food microorganisms. High microbial counts, especially fermented foods are unavoidable lead to BAs accumulation including cadaverine, histamine, putrescine and tyramine (Suzzi and Gardini, 2003; Coton *et al*., 2010; Talon and Leroy, 2011). The BAs content in food products differently depend on many factors such as the qualitative or quantitative composition of microflora, chemico-physical variables, the hygienic procedure applied during fermentation, the precursors availability (i.e., free amino acids) as well as the favorable conditions for growth and decarboxylation (i.e. pH,

temperature and salinity) (Gardini *et al*., 2001; Calzada *et al*., 2013; Latorre-Moratalla *et al*., 2014). The BAs are causing health problems in consumer, especially to sensitive people because of their toxicological effects including nervous, headache and blood pressure problems when excessive consumption (Suzzi and Gardini, 2003; EFSA, 2011a).

Several studies reported that many LAB have the ability of BAs production including enterococci, lactococci, lactobacilli, streptococci, weissella species (Öner *et al*., 2004; Coton *et al*., 2010; Kim and Kim, 2014; Domínguez *et al*., 2016). Jeong and Lee (2015) found that 26% and 44% of *Leuconostoc* and *Weisella* strains isolated from kimchi produced one or more types of BAs in varying amount. This obtained result was in accordance with the report of Tsai *et al*., (2005) and Kim and Kim (2014) who demonstrated that LAB and other microflora growing in kimchi products had the capability to form histamine as well as tyramine. However, the ability of synthesizing BAs is strains-dependent as described by several authors (Garcia-Moruno *et al*., 2005; Landete *et al*., 2011; Jeong and Lee, 2015). The identification of BAs-producing LAB can be examined by the biochemical and genetic techniques. Their ability to decarboxylate amino acids to BAs *in vitro* are reported by previous studies (Landete *et al*., 2011; Kim and Kim, 2014).

9.5.2 Technological properties for starter culture

The starters have to ensure the success of the fermentation process, make the predictability of its product as well as guarantee the quality of the final product (Holzapfel, 2002; Speranza *et al*., 2017). The criteria selection of starter culture is a complicated process including at least 3 different stages: (1) technological and probiotic properties; (2) validation at laboratory scale (process optimization); and (3) validation at industrial scale (Carnevali *et al*., 2007; Bevilacqua *et al*., 2012). A starter culture accepted as QPS status as described by EFSA (2007) should be able to perform the functional properties such as proteolytic and lipolytic enzyme, rapid acid production, salt tolerance, antagonistic activity, reduction of toxic compounds and production of desirable sensory qualities in the fermented products (Holzapfel, 2002; Corbo *et al*., 2017)

Table 3 Microbiological cut-off (μg/ml or mg/l).

Strains	Antibiotics												
	Ampicillin	**Vancomycin**	**Gentamycin**	**Kanamycin**	**Streptomycin**	**Erythromycin**	**Clindamycin**	**Tetracycline**	**Chloramphenicol**	**Tylosin**	**Ciprofloxacin**	**Colistin**	**Fosfomycin**
Lactobacillus obligate homofermentative[a]	2	2	16	16	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus acidophilus group	1	2	16	64	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus obligate heterofermentative[b]	2	n.r.	16	64	64	1	4	8	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus reuteri	2	n.r.	8	64	64	1	4	32	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus facultative heterofermentative[d]	4	n.r.	16	64	64	1	4	8	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus plantarum* / *pentosus	2	n.r.	16	64	n.r.	1	4	32	8	n.r.	n.r.	n.r.	n.r.
Lactobacillus rhamnosus	4	n.r.	16	64	32	1	4	8	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus casei* / *paracasei	4	n.r.	32	64	64	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Bifidobacterium	2	2	64	n.r.	128	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Pediococcus	4	n.r.	16	64	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Leuconostoc	2	n.r.	16	16	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Lactococcus lactis	2	4	32	64	32	1	1	4	8	n.r.	n.r.	n.r.	n.r.
Streptococcus thermophilus	2	4	32	n.r.	64	2	2	4	4	n.r.	n.r.	n.r.	n.r.
Enterococcus faecium	2	4	32	1,024	128	4	4	4	16	n.r.	n.r.	n.r.	n.r.
***Corynebacterium* and other Gram-positive**	1	4	4	16	8	1	4	2	4	n.r.	n.r.	n.r.	n.r.
Enterobacteriaceae	8	n.r.	2	8	16	n.r.	n.r.	8	n.r.	n.r.	0.06	2	8

n.r.: not required.

[a]Including *Lb. delbrueckii, Lb. helveticus*.

[b]Including *Lb. fermentum*.

[c]For *Lb. buchneri* the cut-off for tetracycline is 128 μg/ml.

[d]Including the homofermentative species *Lb. salivarius*.

Source: modified from EFSA (2018)

9.5.2.1 Rapid and adequate production of lactic acid

LAB are able to produce organic acids (mainly lactic acid) from carbohydrates in fermented products. The organic acid production varies significantly depending on the bacterial stains and culture medium (Corbo *et al*., 2017). The acidification by LAB starter participated in the coagulation of muscle proteins that were the resulting in the increased firmness and cohesiveness of the fermented meat products. The organic acids can be reduced the changes of nitrites to nitric oxide to react with myoglobin to form nitrosomyglobin that also enhance pink color of fermented meat during fermentation process (Ammor and Mayo, 2007; Castro *et al*., 2011). Additionally, they contributed to the taste and aroma in final products that gave the noticeable acidic (sourness) (Ammor and Mayo, 2007). The acidify ability of LAB starter culture could prohibit food-borne pathogenic and spoilage bacteria. This statement was in agreement with several studies reported that the inhibition of pathogenic and spoilage bacteria also depend on a rapid and adequate production of these organic acids (Heo *et al*., 2012; Fernandez *et al*., 2013; Speranza *et al*., 2017). The inhibitory effect of organic acids caused by an inactivation of cell membrane transport and various metabolic functions of Gram-positive and Gram-negative pathogenic (Özcelik *et al*., 2016). Moreover, it has been reported that a rapid decrease in pH caused by LAB starter could reduce BAs accumulation in sausage (Latorre-Moratalla *et al*., 2010). Zhang *et al*. (2013) and Sun *et al*. (2016) found that a rapid lowing pH by amine-negative LAB starter was significantly effect on tyramine contents in fermented sausage. Therefore, the rapid production of acids is the primary criteria to select the starter culture for improving quality and safety of fermented products (Ammor and Mayo, 2007; Speranza *et al*., 2017).

9.5.2.2 Growth rate at different salt concentrations and pHs.

The capability of the starter culture should compete with the natural microflora of raw material as well as survive in the conditions prevailing in fermentation processing. The growth rate at different temperatures (2-4°C to 24°C), the tolerance of salt concentrations of 2-10% and of pHs in the range 4.2-6.0 are limiting factors to be affected the persistency and competitiveness of the starter culture during fermentation and ripening process (Ammor and Mayo, 2007; Speranza *et al*., 2017).

Acid tolerance ability is important not only for resistance in gastric condition, but it also enables the strain to survive for longer periods in high acid carrier

foods, such as yogurt, kimchi and fermented sausage without reduction in their amount (Wang *et al*., 2010). Acification ability is elating with the growth of starter culture at low pH conditions in accordance with result of Spernza *et al*. (2017) showed that the growth of selected LAB isolates was severely affected by acidic condition; at pH 2 no isolates was able to grow, whereas at pH most of the targets showed a moderate-to-strong inhibition. Xiong *et al*. (2014) reported that *Lc. lactis* were radpidly grow and produce lactic acid earlier, but poorly acid-resistant, which was similar to the finding by Tolonen *et al*. (2004).

Salt had an affecting on salty taste, flavor, overall acceptance as well as microbial of fermented foods. At salt concentration (2-15%), the starter culture have to tolerate of low water activity (a_w = 0.85-0.98) in the final product. The high level of salt in fermented food can better inhibit the growth of spoilage and pathogenic bacteria (Corbo *et al*., 2017). Previous studies found that some strains of *Leuconostocs* were intolerate towards salt (Di Cagno *et al*., 2009; Wouters *et al*., 2013), which was similar finding by Jampaphaeng *et al*. (2017) who reported that an increasing salt concentration had a negative effect on growth of lactobacilli isolates (KJ03, KJ15, KJ17, KJ22 and KJ23) with a subsequent decrease in cell viability. These results are in agreement with study of Speranza *et al*. (2017) who found that at 7.5% of salt concentration showed moderately-strongly growth inhibition and no growth at over 12.5% salt concentration. Biscola *et al*. (2013) found that *Lc. lactis* 69 was able to survive and produce nisin Z *in vitro* at high salt condition (>20% w/v) to be selected it strain for used as the starter culture in fermented meat (Brazilian charqui). The obtained result demonstrated that addition of *Lc. lactis* 69 in Brazilian charqui could be inhibited the halotolerant of spoilage microbial growth and also prolonged shelf-life of this product (Biscola *et al*., 2014). Additionally, several authors reported that the salinity effected on utilization ratio of sugar and acid production during fermentation (Panagou and Katsaboxakis, 2006; McFeeters and Pérez-Díaz, 2010; Xiong *et al*., 2016). Consequently, LAB could be tolerated salt at least 5% that was considered to be necessary for potential starter cultures in fermented products (Ammor and Mayo, 2007; Settanni abd Corestti, 2008; Xiong *et al*., 2014).

10. Microbial community of fermented food

The main components of the fermentation ecosystem include: microbes (bacteria and yeast), organic material to be fermented, a solution in which the fermentation takes place, a vessel with a controlled gate, and various tools which may be used to develop and monitor the fermentation (thermometer, hydrometer, siphoning tubes, etc.). This is an ecosystem in that it is a complex of living and non-living components that are viewed in terms of their interactions in a specific place. Knowledge of microbial metabolism is not necessary for the production of fermented foods, and indeed wasn't even proposed until 150 years ago.

Today, all human cultures rely on fermented food products and the microbes that produce them. Fermentation is not only a component of human ecology, but also has an ecology of its own. Over the past 150 years, microbiologists have uncovered this unseen world of ecosystems that have always lived in our food. A food ecosystem is not static. The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to changing physical and chemical conditions that occur in the food micro-environment (e.g. pH, salt, temperature), the ability of microorganisms to colonize the food matrix and to grow into spatial heterogeneity (e.g. micro-colonies and biofilms), and the *in situ* cell-to-cell ecological interactions which often happen in a solid phase. Reliable quantitative microbiological data should, therefore, take into consideration the dynamics of microorganisms in food ecosystems. This information is of key importance in food ecology, especially in understanding the behavior of pathogens and LAB in foods (Cocolin *et al*., 2004; Rantsiou and Cocolin, 2006; Randazzo *et al*., 2012; Perin *et al*., 2015).

To this regard, estimates of true microbial diversity in fermented food products are often difficult chiefly on account of the inability to cultivate most of the viable bacteria. The traditional methods of microbial enumeration, identification, and characterization are deficient for monitoring specific strains or mixed strains in matrix foods and/or environment. In the last decade, due to the use of molecular methods, our knowledge about the microbial diversity of microbial ecosystems has dramatically increased. In particular, new and highly performing culture-independent and culture-dependent molecular techniques are now available to study food-associated microbial

communities. While the former is helping to afford peculiar problems related to composition and population dynamics of heterogeneous microbial communities in complex food matrices, the latter is expanding our knowledge about taxonomic diversity of the food-related microflora. Molecular approaches to study the evolution of microbial flora could be useful to better comprehend the microbiological processes involved in food processing and ripening, improve microbiological safety by monitoring *in situ* pathogenic bacteria, and evaluate the effective composition of the microbial populations (Ercolini, 2004; Fontana *et al*., 2005; Cocolino and Ercolini, 2008; Fujii *et al*., 2011; Liu *et al*., 2017).

10.1 Molecular techniques to study of microbial community of food fermentation

10.1.1 Target tools

The targeted molecular profiling method is technique to detects a taxonomically identified group of microbial, e.g., a specific species or a specific strain. The principal of all targeted methods can only quantify populations targeted by the selected probes or primers. However, these techniques cannot detect non-target microbial populations and cannot differentiate subpopulations (without using additional probes). Therefore, these techniques are not comprehensive profiling techniques (i.e., they do not identify a complete microbial ecology), but are determined to observe important members of a given ecosystem.

10.1.1.1 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR, also known as real-time PCR) has become a widespread technique for food microbiology determining and microbial ecosystem studies. The qPCR consolidates the principles of DNA replication used by PCR technique. The amplification is monitored in real-time via a fluorescent reporter molecule. A baseline threshold is measured, at which sample fluorescence can be differentiated from background noise. This technique determined the threshold cycle (Ct) of each sample, which fluorescence crosses the baseline threshold. A standard curve is built plotting Ct against cell count or copy gene number that quantified unknown samples based on Ct. The result is simple, rapid and also accurate means of monitoring gene counts or target cell in samples. The qPCR has been widespread use for enumeration of microbial populations in foods (Postollec *et al*., 2011). Several researchers have been used qPCR to detect BAs-producing LAB in wines (Lucas *et al*.,

2008), ciders (Ladero *et al*., 2011) and cheeses (Fernández *et al*., 2006; Ladero *et al*., 2010) by directly targeting the genes involving BAs production. Ibarburu *et al*. (2010) developed the method for quantification of LAB produced exopolysaccharide (EPS) which cause slime-production (a defect in wines and other beverages) by determining the glycosyltransferase gene regulating EPS synthesis. Additionally, qPCR method has been tracked the sulfide production that was a major off-flavor concern in wine fermentations (Mendes-Ferreira, 2010). Another novel application of qPCR is the use of strain-specific primers/probes to identify the strain-level successions in food fermentations (Cho *et al*., 2011).

10.1.1.2 Fluorescence *in situ* hybridization (FISH)

FISH is a culture-independent, the technique can directly visualize microbial counts in a sample. This method uses the fluorescence-labeled oligonucleotide probes to be targeted specific DNA sequences. Multiple probes can be multiplexed to enable simultaneous enumeration and detection of many targets in microbial communities by labeled probes with a different fluorophore that exhibits a unique emission spectrum. Cells are fixed and permeabilized *in situ*, incubated in the presence of the probes to facilitate hybridization, and observed directly by fluorescence microscopy or counted using flow cytometry. This technique is widespread for monitoring microbial communities, it can be avoided the biases and challenges of culturing and PCR. In additional, this technique can be observed the target cells within their native environment, a feature with exciting applications to food fermentations. The major issues with using FISH for microbial ecology is not an efficient for counting cells when comparison with quantitative PCR (qPCR) due to the probes may be overlapped the emission wavelength (Andorra *et al*., 2011).

FISH has been used to monitor microbial communities in foods such as wine (Andorra *et al*., 2011; Wang *et al*., 2014), dairy products (Bottari *et al*., 2010; Machado *et al*., 2013), meat (Vieira-Pinto *et al*., 2008; Shimizu *et al*., 2009). Shimizu *et al*. (2009) designed a FISH protocol for *in situ* enumeration of *C. perfringens* in food sample. The result presented that FISH technique was equally accuracy on the detection limit of 2 log CFU/g and was faster than conventional plate count method. Additionally, FISH has been used for other studies of microbial distribution in a solid matrix, e.g., oral biofilms (Klug *et al*., 2011). However, FISH is not a stand-alone method for microbial detection, it exhibits a compelling complementary method to visualize

biogeography of microbial communities in sample. Moreover, FISH technique is suitable for the study of fermented foods, especially those employing heterogeneous and fermented food products (Vieira-Pinto *et al*., 2008; Shimizu *et al*., 2009; Andorra *et al*., 2011).

10.1.2 Community profiling tools

The culture-independent methods for microbial ecology used the universal primers targeting microbial group for amplifying heterogeneous DNA sequences from mixed microbial samples before separating and sequencing. This technique provides a qualitative (relative abundance) evaluation of a microbial community.

10.1.2.1 Terminal restriction fragment length polymorphism (TRFLP)

TRFLP is widespread technique for studying microbial ecosystem, but has received much less attention by food microbiologists. This method distinguishes microbial populations based on terminal restriction fragment (TRF) size. Mixed DNA samples were amplified with fluorescence-labeled universal primers. After digesting PCR amplicons with select restriction enzymes, these samples were separated by capillary electrophoresis with molecular standards for estimating fragment size. Only the labeled 5' terminal fragments are detected and compared to a database to determine which bacterial populations (grouped as operational taxonomic units, OTUs) are represented by these fingerprint-like markers. Fluorescence intensity is integrated to pseudoquantitatively determine OUT abundance relative to total fluorescence.

TRFLP is prone to the same amplification-related biases common to all PCR-based techniques. In addition, as electrophoretic mobility is a function of molecular weight, charge, and sequence structure, both sequence variability and fluorescent labels may alter the electrophoretic migration of TRFs, resulting in a reproducible disparity between expected and observed TRF sizes (Kaplan and Kitts, 2003; Marsh, 2005). This problem may be ameliorated by creating an empirical TRFLP database for the microbial species expected in a sample, but this process is time-consuming and can make critical assumptions about the microbial constituents of a given system. Residual polymerase activity (Hartmann *et al*., 2007) and single-stranded "pseudo-TRFs" (Egert and Friedrich, 2003) can also create artificial OTUs, though both of these errors can be corrected by proper procedural modification. The largest issue with TRFLP, however, is that sequence homology among related microbial taxa limits

the taxonomic depth achieved using this technique, so a single TRF is identified to the common taxon encompassing all possible database matches. The shallow taxonomic depth often achieved by universal primers may be increased to species-level by using clade-specific primers, for example specific for LAB (Bokulich and Mills, 2012). TRFLP has several advantages that suit it for microbial com- munity profiling compared to DGGE. Most importantly, TRFLP is a high-throughput, automatable process, unlike DGGE, so can compare much larger sample sets rapidly and efficiently. Thus, TRF profiles may be integrated and used for diversity comparisons between samples on a massive scale. A common approach is to use principle component analysis or a similar approach to cluster samples based on TRF abundance and visualize these differences in 2-dimensional space (Culman *et al.*, 2008). Similarly, TRFLP is commonly used to calculate diversity statistics such as Jaccard distance (though caution is advised, as TRFLP can over-estimate species diversity due to the above-mentioned errors (Blackwood *et al.*, 2007) and ANOVA or other multivariate statistics can compare significance among clusters or treatment categories (Culman *et al.*, 2008). Additionally, TRFLP is a pseudoquantitative method that calculates relative species abundance based on sensitive capillary electrophoresis separation and fluorescence detection, while DGGE is not, based on polyacrylamide gel separation and UV detection.

TRFLP has become a popular technique for microbial profiling (Schütte *et al.*, 2008) but has been little-used for studying food systems. Rademaker *et al.* (2005) first applied TRFLP to study the microbiota in cheese and yogurt fermentation. Others have applied reverse-transcription TRFLP to analyze viable bacterial communities during cheese fermentations (Sánchez *et al.*, 2006). Specific TRFLP assays have been developed for species-level differentiation of LAB (Bokulich and Mills, 2012) in fermented foods and beverages, and used to profile microbial successions during beer (Bokulich and Mills, 2012) and wine fermentations (Bokulich *et al.*, 2012). TRFLP has also been used to analyze the bacterial composition of commercial probiotic products (Marco *et al.*, 2008). This technique shows much promise as a low-tech, high-throughput method for studying microbial ecology, but has been largely overlooked by food microbiologists. With the advent of next-generation sequencing tools, TRFLP remains a viable complementary method for exploring species-level dynamics.

10.1.2.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE is molecular technique that attended from food microbiologists in the past 20 years since it was first adopted for detecting bacterial communities (Muyzer *et al*., 1993). DGGE is closely relative to temperature gradient gel electrophoresis (TGGE) that separates the short DNA fragments along a chemical or temperature gradient, respectively. DNA passed through a polyacrylamide gel matrix by an electric current until it reaches the point along the gradient at DNA destabilized or partially denatures (held together by a high-stability GC tail) to be immobilized in the gel (Fig. 12). The abundant of these fragments visualized on the gel under UV light, physically extracted, reamplified, and sequenced to qualitatively determine the most dominant organisms in a given sample. However, DGGE is hindered by several issues that make it unsuitable for large-scale studies of microbial ecology. First of all, DGGE is a time-consuming and technically challenging method. Additionally, the denaturing gradient gels are difficult to set and run properly. DGGE only serves to separate DNA fragments, and a representative band from each position on a gel must be extracted and sequenced to identify the closest match. However, only a small number of samples may be run on a single gel, and so representative bands must be sequenced from each, making analysis of large sample sets time-consuming, redundant, and expensive. Band co-migration (Sekiguchi *et al*., 2001; Kisand and Wikner, 2003) and rRNA gene multi-copy heterogeneity (Nübel *et al*., 1996) further complicate this process, respectively hindering band purity and increasing redundancy. Another problem with this method is that it is non-quantitative, so can detect the presence of the dominant species present but cannot reliably determine relative abundance, though others dispute this point, suggesting integration of band size and intensity against a densitometric curve may be an accurate indicator of abundance (Tourlomousis *et al*., 2010).

Several studies have been used DGGE technique to pioneer the microbiota in food system (Sanchart *et al*., 2015; Diaz *et al*., 2016) and investigate the endodontic infection (Siqueira *et al*., 2005). Diaz *et al.* (2016) used DGGE technique to identify the histamine-producing bacteria in cheese. This result showed that DGGE based method could identify the histamine-producing bacteria, at the species level in cheese ripening. Sanchart *et al*. (2015) applied *rpoB* gene amplicons to cooperate with DGGE method for studying the profile of bacterial population in *kung-som*.

Consequently, the bacterial population in *kung-som* product was demonstrated that *Lb. farciminis*, *Lb. plantarum* and *Tetragenococcus halophilus* were predominant bacteria. Additionally, DGGE technique has been used to investigate the profile of microbiota in *Shenqu* using different fermentation time periods. The DGGE result demonstrated that *P. acidilactici*, *Aspergillus oryzae* and *Rhizopus oryzae* were dominant microbial and declined over the periods of fermentation (Liu *et al*., 2017).

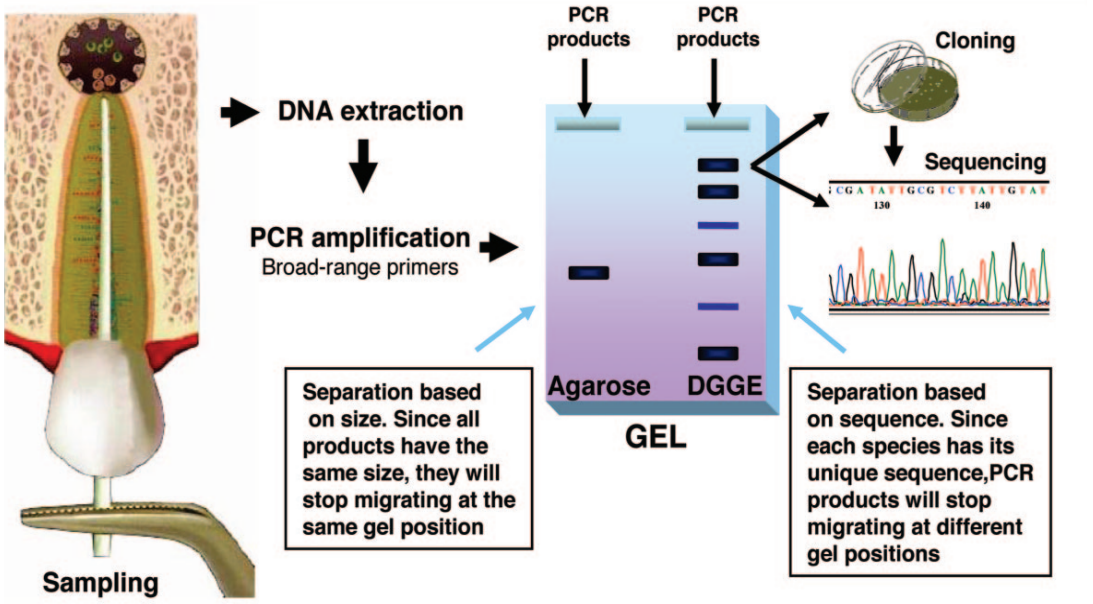

Figure 12 Scheme for PCR-DGGE technique in endodontic microbiota.
Source: Siqueira *et al*. (2005)

Nalepa and Markiewicz (2017) successfully developed reference markers for the qualitative evaluation of microbial diversity in dairy products by PCR-DGGE based method. The developed makers could identify LAB species including *Leuconostoc* spp. *L. brevis* and *Lb. plantarum* in raw milk. Moreover, reference makers demonstrated *Lc. lactis*, *Leuconostoc* spp. and *P. freudenreichii* in chees. The result suggested that developed markers are a useful tool for identification of the microbiota of raw milk and cheese samples, which reduced the time and cost of analysis. Furthermore, Reverse Transcriptase-PCR-DGGE techniques (RevT PCR-DGGE) was developed for rapid detection of pathogenic in foods.

For the diagnostic of disease infection, DGGE based method can be contributed the microbiological diagnosis of diabetic foot ulcers. The study of Dunyach-Remy *et al*. (2014) showed that DGGE technique could be identified the

pathogenic bacteria species (Enterobacteriaceae, β-haemolytic streptococci, *S. aureus* and *Proteus mirabilis*) relating foot infections more than conventional method. This method could be diagnosed the bacteriological of foot infection in 48 h. It was useful tool to rapid identify the pathogen in deeply infected ulcers for contributing to a more appropriate use of medicine. De Paula *et al*. (2014) found that PCR-DGGE analysis demonstrated *Atopobium rimae*, *Staphylococcus* sp., *Campylobacter* spp., *Johnsonella* sp. and uncultured bacterium responsible for endodontic infection. This technique revealed to be useful for evaluation of endodontic microbiota in root canal with necrosis and also selected the most effective medication.

Objectives of study

1. To assess the safety of *kung-som* products sold in local markets and identify the dominant bacterial species in the product of *kung-som* using PCR-DGGE technique.
2. To isolate, purify and characterize bacteriocin-producing LAB isolated from Thai traditional fermented shrimp (*kung-som*) and evaluate the safety characteristics and effectiveness of selected bacteriocin-producing strain to control *S. aureus* in co-cultivation.
3. To screen the tyramine-producing bacteria isolated from *kung-som* by chromatographic and molecular methods and evaluate the effectiveness of the inhibition of tyramine-producing bacteria and reduction of tyramine accumulation *in vitro* and *in situ* by selected bacteriocin-producing strain.
4. To evaluate the safety aspects and technological properties of CNS isolated from *kung-som* and study the effect of inoculating selected CNS strain and selected bacteriocin producing strain as starter culture on the physicochemical, microbiological, sensory properties and volatile profile of *kung-som*.

CHAPTER 2

SAFETY EVALUATION AND BACTERIAL COMMUNITY OF *KUNG-SOM* USING PCR-DGGE TECHNIQUE

2.1 Abstract

The aim of this study was safety assessment of a traditional Thai fermented shrimp (*kung-som*) which distributed in local markets and identification of microbial community of *kung-som* using PCR-DGGE technique. For safety evaluation, all samples of *kung-som* did not detect the pathogenic bacteria including *Escherichia coli*, *Clostridium perfringens*, *Staphylococcus aureus* and *Salmonella* sp. Lactic acid bacteria (LAB) was predominant bacteria that found more than 7.0 log CFU/g and total viable counts (TVC) were in range of 5.2-8.6 log CFU/g. DGGE analysis demonstrated that diverse LAB belonging to the genera *Lactobacillus*, *Lactococcus*, *Tetragenococcus* and *Weissella* were the dominant bacteria in *kung-som*. Additionally, the obtained result from DGGE showed coagulase-negative staphylococci (CNS) as a minor microflora in *kung-som*. These finding suggested that understanding of bacterial community presented in *kung-som* and providing a theoretical basis to develop the starter cultures for improving the quality of *kung-som* product.

2.2 Introduction

Kung-som is a traditionally Thai fermented shrimp, which is widely consumed in southern Thailand. It is generally made by mixing 7% (w/w) salt and 30% (w/w) sugar to shrimp and fermenting until obtained sour-tasting in product. The recipes of traditional fermented foods varied by region, depending upon the ingredients available and local consumer preferences. Lactic acid bacteria (LAB) play an important role in the *kung-som* fermentation, and are found in raw materials and ingredients, and in the local atmosphere (Kopermsub and Yunchalard, 2010). To date, that studied taxonomies of the LAB isolated from fermented shrimp use the cellular fatty acids analysis and carbon source utilization profiles. On the other hand, the results obtained with these different assays of analysis are not directly comparison.

Currently, cultivation methods are limit for detection and identification of microbiota that involved in fermentation of food. This method did not provide reliable information on the composition of the entire microbial community (Randazzo *et al*., 2012). Several studies have used the culture-independent technique to evaluate the microbial ecology in foods (Ercolini, 2004) such as fermented sausages (Fontana *et al*., 2005), fermented fish (Fujii *et al*., 2011) and kimchi (Lee *et al*., 2005). Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons has been demonstrated to be a potential tool for rapid detection microflora species composition in fermented products. However, there are no previous report of bacterial communities in *kung-som*. Therefore, the aims of the present study were to assess the safety of *kung-som* products sold in local markets, and to identify the dominant bacterial species in the finished product of *kung-som*. This study was done using a molecular approach combining PCR-amplification of the V3 region of the 16S rDNA and DGGE technique.

2.3 Materials and Methods

2.3.1 *Kung-som* samples

Eighteen samples of *kung-som*, sold as a commercial product, were bought from randomly selected vendors scattered over different markets in Songkhla Province. The samples were transported to the laboratory for analysis.

2.3.2 Microbiological analysis

The microbiological analysis of *kung-som* was based on the methods described in Biological Analysis Manual (BAM, 1998) and Thai Community Product

Standard (TCPS, 2014). Representative samples (25 g) were transferred into 225 ml sterile peptone water (0.1% (w/v) peptone; Hi-media, India) and homogenized using a stomacher machine at 230 rpm for 1.5 min. The samples were further diluted in a 10-fold serial dilution and 100 µl of the appropriate dilutions were spread-plated on plate count agar (PCA; Hi-media, India) for the total viable count and on de Man-Rogosa-Sharpe (MRS) medium for LAB (Lab M, England). The cultures on the PCA and MRS medium were incubated at 35°C for 24-48 h.

The enumeration of *Bacillus cereus* and *Staphylococcus aureus* was done by spread-plating on mannitol egg yolk polymixin agar (Hi-media, India), and Baird-Parker agar (Hi-media, India) and incubated at 35°C for 24-48 h, respectively. The *Clostridium perfringens* was pour-plated using tryptose sulfite cycloserine agar (Merck, Germany), and incubated at 35°C under an anaerobic condition for 24-48 h. Total coliform bacteria and *Escherichia coli* were enumerated using the 3-tubes Most Probable Number (MPN) method. *Salmonella* sp. from each sample was applied at certain intervals according to the method described by BAM (1998).

2.3.3 pH and acidity measurement

The pH of undiluted juice samples was measured using a pH meter (420A ORION; Thermo Scientific, MA, USA). The determination of lactic acid (%) in *kung-som* was determined by as described by Association of Official Analytical Chemists (AOAC, 2002).

2.3.4 Extraction of bacterial DNA from *kung-som*

Ten grams of the sample were homogenized in a stomacher bag with 90 ml of peptone water for 1.5 min. The debris was allowed to precipitate for 1 min. Genomic DNA was extracted as previously described by Cocolin *et al*. (2004) with some modifications. For the first step, the supernatant was centrifuged at 6,700×*g* at 4°C for 10 min. The cell pellets were resuspended in 50 µl of lysozyme (20 mg/ml; Sigma-Aldrich, MO, USA) and incubated at 37°C for 30 min. Then 30 µl of proteinase K (25 mg/ml; Amresco, OH, USA) in 150 µl of proteinase K buffer (50 mM Tris-HCl; 10 mM EDTA, pH 7.5; 0.5% w/v, sodium dodecyl sulfate) were added and incubated at 65°C for 60 min. Then, 400 µl of breaking buffer (4% v/v, Triton X-100; 2% w/v, sodium dodecyl sulfate; 200 mM NaCl, 20 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0) was added and mixed by tube inversion. Four hundred microliters of phenol/chloroform/isoamyl alcohol (25/24/1 v/v/v; Amresco, USA) was added for

protein elimination and mixed by tube inversion. The tubes were centrifuged at 11,300×*g* at 4°C for 15 min and the aqueous phase was precipitated by adding 1 ml ice-cold isopropanol and then centrifuged at 11,300×*g* at 4°C for 15 min. Then the pellet was washed with 200 µl of 70% (w/v) alcohol and centrifuged at 11,300×*g* at 4°C for 15 min. The genomic DNA was dried under room temperature for 15 min. Finally, the genomic DNA was resuspended in 30 µl of sterile MiliQ water and kept at -20°C.

2.3.5 PCR amplification of V3 region of 16S rDNA and DGGE analysis

Genomic DNA was used as a template material to amplify the V3 region of the 16S rDNA by PCR using the primers 341f (5′-CCTACGGGAGGCAGCAG-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993). PCR reaction was performed in 50 µl containing 0.2 µM of each primer, 25 µl of GeNei™ Red Dye PCR Master Mix (Merck, Germany), DNase free water 14 µl and 1 µl of template DNA. The PCR conditions was performed by using a thermal cycler TC-5000 (Techne, England) with an initial denaturing of 3 min at 95°C, followed by 25 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C with a final extension of 10 min at 72°C. Amplicon was analyzed by electrophoresis on 2% (w/v) agarose gel containing 1×SYBR Safe (Invitrogen™, CA, USA) and then visualized by UV transillumination with the GelDoc (UVItech, England).

For DGGE analysis, the PCR products of V3 region were reamplified with primer 341f-GC (5′-CGCCCGCCGCGCGCGGGGCGGGGCGGGGGCACG GGGGGCCTACGGGAGGCAGCAG-3′) (Muyzer *et al*., 1993). The PCR program used an initial denaturing for 3 min at 95°C; followed by 25 cycles of 30 s at 94°C, 1 min at 65°C, 30 sec at 72°C; and finally, 10 min at 72°C. Sequence specific separations of PCR amplification products were performed by using an OmniPAGE Maxi Electrophoresis Systems VS20-DGGE (Cleaver Scientific, England). PCR samples (~40 µg) were loaded on the DGGE gels that were formed with 8% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide, ratio: 37.5/1; Amresco, USA) using a denaturant gradient 28-55% (w/v). The electrophoresis operation was performed at 20 V for 10 min and then 85 V for 15 h in 1×TAE buffer maintained at 60°C. After staining the gel with 1×SYBR Gold (Invitrogen™, USA) for 30 min, the gel was rinsed for 5 min in MiliQ water and photographed with the GelDoc.

2.3.6 Excision and sequencing of the DGGE fragments and phylogenetic analysis

The bands were cut from the DGGE gels by using sterile scalpels and punched with pipette tips. The DGGE bands were resuspended in 20 µl of sterile MiliQ water and stored overnight at 4°C as previously described by Fontana *et al*. (2005). An aliquot of eluted DNA was used for reamplification with the forward primer without the GC-clamp. DNA fragments were purified using a HiYield Gel/PCR Fragments Extraction Kit (RBC, Taiwan). The purified PCR fragments were sent to the 1st BASE company (Kuala Lumpur, Malaysia) for sequencing. DNA sequences were analyzed using Blastn of NCBI nucleotide to evaluate the closet known relatives of the partial 16S rDNA sequence deposited in the GenBank database (Tamura *et al*., 2011).

2.4 Results and Discussion

2.4.1 Microbiological safety

Kung-som, an indigenous salted shrimp, is characterized by acidity with a final pH of lower than 4.6 (TCPS, 2014). The microbiological safety of *kung-som* is of major importance to consumers and food industry. Generally, *kung-som* is not heated or boiled before consumption that is risk of foodborne illnesses due to organic acids in fermentation process that might not inhibit all the pathogenic bacteria, which are found in raw materials. This study therefore offers the first of information about the microbial status of traditionally fermented shrimp in southern of Thailand. A total of 18 samples of *kung-som* bought from different local markets in Songkhla Province, Thailand. They were analyzed for microbiological safety based on the TCPS (2014) as shown in Table 4. The microbial population was predominated by LAB (>7 log CFU/g). Various authors reported LAB as microflora in Thai fermented products (*pla-ra*, *plaa-som, kung-chom*) (Tanasupawat, 2009; Kopermsub and Yunchalard, 2010).

Table 4 Microbiological characteristic of *kung-som* collected from local markets in Songkhla Province.

Samples	**Microbiological analyses (log CFU/g)**							
	TVC	LAB	*S. aureus*	*C. perfringens*	*B. cereus*	Total coliform (MPN/g)	*E. coli* (MPN/g)	*Salmonell*a sp.
A1	6.9	8.5	nd	nd	nd	<3	nd	nd
A2	6.6	7.9	nd	nd	nd	<3	nd	nd
A3	5.4	7.0	nd	nd	1.5	<3	nd	nd
A4	5.5	8.6	nd	nd	1.5	<3	nd	nd
A5	8.2	7.9	nd	nd	1.3	<3	nd	nd
A6	5.7	8.0	nd	nd	1.3	<3	nd	nd
A7	5.6	7.5	nd	nd	1.3	<3	nd	nd
A8	5.8	8.3	nd	nd	1.5	<3	nd	nd
A9	5.2	7.7	nd	nd	1.3	<3	nd	nd
A10	6.7	8.1	nd	nd	nd	<3	nd	nd
A11	5.5	8.2	nd	nd	1.3	<3	nd	nd
A12	5.8	8.1	nd	nd	1.5	<3	nd	nd
A13	8.6	8.8	nd	nd	nd	<3	nd	nd
A14	5.8	8.5	nd	nd	nd	<3	nd	nd
A15	5.6	8.2	nd	nd	1.5	<3	nd	nd
A16	6.7	8.1	nd	nd	1.8	<3	nd	nd
A17	6.4	7.8	nd	nd	1.5	<3	nd	nd
A18	7.5	8.5	nd	nd	2.0	7.2	nd	nd
TCPS	-	-	<2.0	nd	<3.0	-	<3	nd

nd: non-detectable.

TCPS: Thai Community Product Standard (2014) (no. 1032/2014).

Number of total viable counts (TVC) ranged from 5-8 log CFU/g that was similar to results (4-8 log CFU/g) in a fermented shrimp (*kung-chom*) (Prachasitthisak and Eamsiri, 2009). Lee *et al*. (2002) reported that the number of TVC of salted (15% and 20%) fermented shrimp (*jeotkal*) was 5 log CFU/ml. Count of total coliforms on samples showed low bacterial population. Pathogenic bacteria, such as *C. perfringens*, *E. coli*, *S. aureus* and *Salmonella* sp., did not detect in any of the samples, which caused by the reduction of pH <4.2 (Fig. 13) through the lactic acid as major organic acid produced by LAB. Accordingly, the amount of lactic acid in samples presented in the range from 1.4-3.5% (Fig. 13) and sufficiently inhibit the food-borne pathogenic and food spoilage bacteria. This result was in agreement with study of Wang *et al*. (2014) found that 1.0% and higher lactic acid concentration showed more effective to inhibit the pathogens growth. It has been proposed that the un-dissociated form of organic acid reduced the pH in the cytoplasm that influenced to decrease the intracellular pH, inhibiting essential microbial metabolic reactions (Olasupo *et al*., 2004; Zhu *et al*., 2006).

Low level of *B. cereus* (2 log CFU/g) was detected in some *kung-som* samples (Table 4). However, it did not exceed the threshold risk level of 4 log CFU/g. EFSA (2006) reported that in most instances, foodborne diseases caused by *B. cereus* were associated with 5-8 log CFU/g. *B. cereus* survived in high acidic condition because of its strain responded to acid stress by forming bacterial spores. Previous study reported that spores of *B. cereus* were tolerant to acidity at pH value between 1-5 at *in vitro* condition (Clavel *et al*., 2004). *B. cereus* did not detect in *kung-som* sample A1 and A3 even though the samples had low lactic acid content (<1.7%) compared with other samples (Fig. 13 and Table 4). This result showed that fermentation process was a good hygiene procedure to control contamination of *B. cereus* in the product of *kung-som.* Additionally, this inhibitory effect might be caused from the dominant LAB produce not only organic acid but also other antimicrobial compounds (hydrogen peroxide, reuterin and bacteriocin) in the products of *kung-som*. Therefore, *kung-som* samples which were collected from Songkhla Province are safe for consumer consumption (TCPS, 2014).

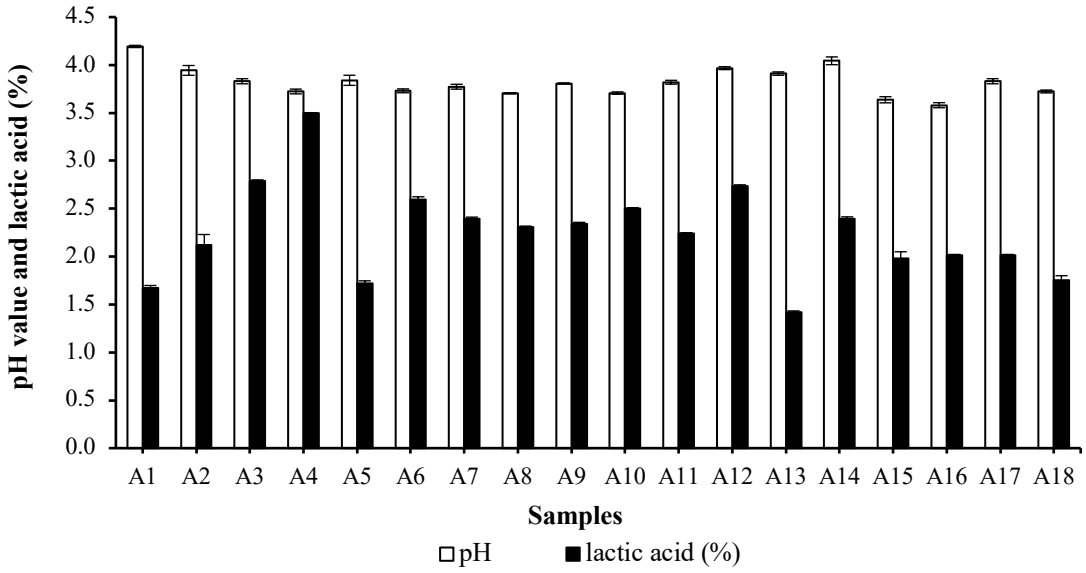

Figure 13 pH and lactic acid (%) of *kung-som* collected from local markets in Songkhla Province. Bars represent the standard deviation from three determinations.

2.4.2 Bacterial community of *kung-som* using DGGE technique

Kung-som fermentation continues to be performed in a traditional manner, resulting in a great bacterial diversity of the final products. In the present study work, a PCR-DGGE technique was used for the first time to systematically study the bacterial communities of Thai traditional fermented shrimp. The fingerprints obtained from the bacterial community in finished products of *kung-som* were analyzed using the DGGE technique, which demonstrated 16 different visible bands as shown in Fig. 14. No difference was detected in the DGGE profiles when replicates obtained from the same samples were analyzed (data not shown). To identify the main DGGE bands, each band was recovered from the DGGE gel and sequenced. These sequences exhibited a greater than 96% identity to the sequences deposited in the databases (Table 5) which were identified as belonging to 7 genera: *Alkalibacterium*; *Enterococcus; Lactobacillus; Lactococcus; Staphylococcus; Tetragenococcus* and *Weissella*.

These LABs were the major component in the bacterial composition of *kung-som*. The bands indicative for *Lactobacillus farciminis* (band 4, 5 and 6) and *Lb. plantarum* (band 12) were observed with a high intensity in *kung-som*, indicating their important role in *kung-som* fermentation. *Lb. farciminis* showed multiple bands in DGGE gels. Ercolini (2004) had been reported that a single species with multiple rDNA

copies could demonstrate maniple bands in a DGGE gel, which overestimates the community diversity detected by PCR-DGGE technique. *Lactobacillus* species, especially *Lb. farciminis* and *Lb. plantarum* have been frequently isolated from various aquatic products such as commercial cold smoked salmon (Tomé *et al*., 2006) and Asian fermented seafood products such as Thai fermented fish and shrimp (Tanasupawat *et al*., 2000; Tanasupawat, 2009), Japanese fermented fish (Fujii *et al*., 2011) and Taiwanese fermented clams (Chen *et al*., 2012). In the present study, *Lb. farciminis* and *Lb. plantarum* were detected in the final stage of the fermentation process using DGGE technique, they are acid-tolerant LAB and can grow in high acidity conditions (Kopermsub and Yunchalard, 2010; Miyashita *et al*., 2012). In addition, *Lactococcus garvieae* (band 13), *Tetragenococcus halophilus* (band 1 and 2) and *Weissella thailandensis* (band 14 and 15) were also identified as significant components of the microflora in *kung-som*.

Lc. garvieae has been previously reported as the strain isolated from fermented fish products (*plaa-som*) during an early stage of fermentation (Kopermsub and Yunchalard, 2010) and it can be isolated from brined shrimp and fermented clams (Dalgaard *et al*., 2003; Chen *et al*., 2012). Several researches reported that *T. halophilus* is a bacterial flora in various fermented seafood products that was detected by culture dependent and culture independent technique (Chen *et al*., 2012; Chuon *et al*., 2014). Moreover, it has been reported that *Weissella* species was found in certain aquatic products (Chen *et al*., 2012). Tanasupawat *et al*. (2000) reported that *W. thailandensis* was the most frequently found *Weissella* species in Thai fermented fish food. From the data of previous studies, it is known that *W. hellenica* was the most abundant LAB in Taiwanese fermented clams (Chen *et al*., 2012). This is the first report of *W. thailandensis* in Thai fermented shrimp *kung-som* by using DGGE technique.

Alkalibacterium kapii (band 7), *Enterococcus faecium* (band 9) and *Pediococcus pentosaceus* (band 8) were found in some samples. As previously described, fermented foods are made differently in different regions owing to location-specific factors such as raw materials for fermentation, ingredients available in the area, climate condition and fermentation methods and there affect the bacterial flora in the product (Lee *et al*., 2005). Literature reports also confirmed the isolation of *Ent. faecium* and *P. pentosaceus* from different marine food products and Thai fermented food (Miyashita *et al*., 2012). *Alkalibacterium* sp. with alkaliphilic and

halophilic properties were isolated from marine organism. Few studies have reported the presence of *A. kapii* in fermented foods. Ishikawa *et al*. (2009) reported that *A. kapii* is a halophilic bacterium isolated from Thai fermented shrimp paste (*ka-pi*), salted fish and raw fish. Among the bacteria isolated from *kung-som*, coagulase-negative staphylococci (CNS) made up the minor part of the community; *Staphylococcus carnosus* (band 3) and *Staphylococcus* sp. (band 11) were detected in DGGE gels. *S. carnosus* are halotolerant bacteria and, as with CNS, their pathogenicities have not yet been reported (Guan *et al*., 2011). They have nitrite and nitrate reductase activity, enhance the flavor of fermented foods, promote the desired red color development and help stabilization, which limits lipid oxidation and prevents rancidity. *S. carnosus* was the dominant detected in fermented sausage and are used as a starter culture in fermented meat products (Fontana *et al*., 2005; Talon *et al*., 2007).

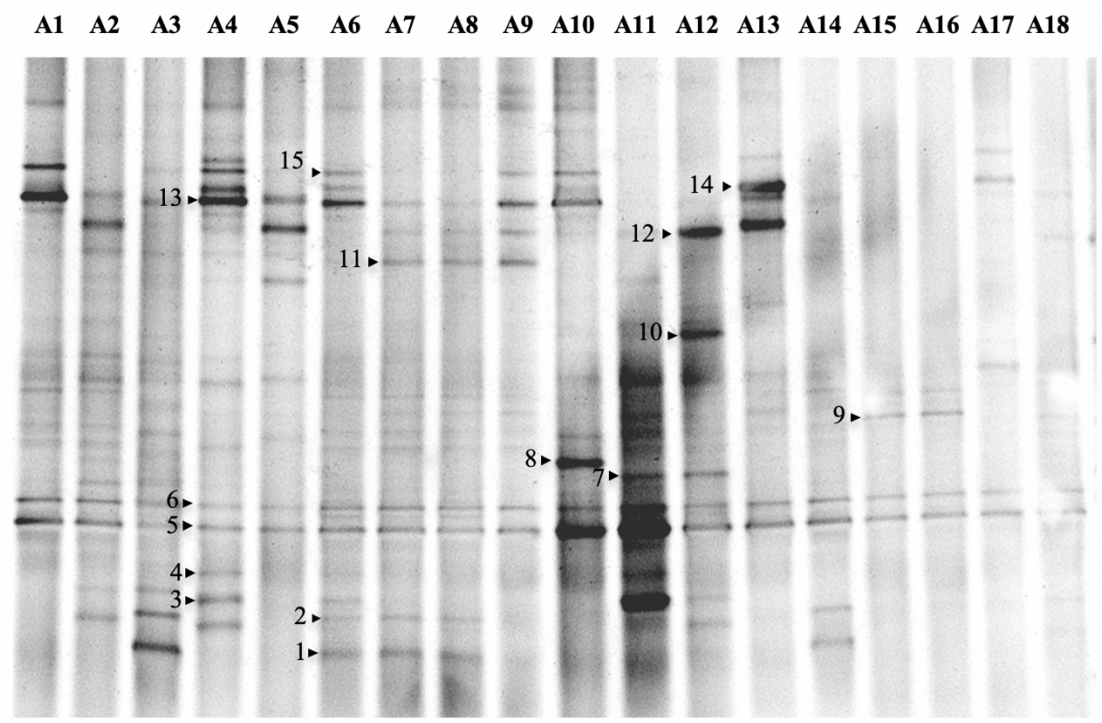

Figure 14 Denaturing gradient gel electrophoresis (DGGE) profiles of DNA amplicons obtained directly from *kung-som*. Sequence of bands (1-15) was searched in the GenBank with the BLAST program to determine the closest known relatives of the partial 16S rDNA sequences obtained (Table 5).

Table 5 Identification of dominant fragments in DGGE patterns in *kung-som* collected from local markets in Songkhla Province.

Band no.[a]	Highest match with accession number in parenthesis	Identity (%)	Accession numbers[b]
1	*Tetragenococcus halophilus*	99	AB681296.1
2	*Tetragenococcus halophilus*	100	AB681296.1
3	*Staphylococcus carnosus*	100	EU727181.1
4	*Lactobacillus farciminis*	98	AB840589.1
5	*Lactobacillus farciminis*	100	AB840589.1
6	*Lactobacillus farciminis*	96	AB840589.1
7	*Alkalibacterium kapii*	97	AB681991.1
8	*Pediococcus pentosaceus*	100	AJ305321.1
9	*Enterococcus faecium*	96	AJ301830.1
10	Uncultured bacterium	98	JN708339.1
11	*Staphylococcus* sp.	99	KC121048.1
12	*Lactobacillus plantarum*	100	AB626055.1
13	*Lactococcus garvieae*	100	EU121676.1
14	*Weissella thailandensis*	100	AB023838.1
15	*Weissella thailandensis*	99	AB023839.1

[a]Band number as indicated on DGGE gels as shown in Fig. 14.
[b]Accession number of the sequence of the closest relative species identified using the Blast software.

2.5 Conclusion

Our result of present study concluded that finished product of *kung-som* collected from Songkhla are safe for consumption according to Thai Community Product Standard. The bacterial flora of the traditional Thai fermented shrimp (*kung-som*) using DGGE analysis. *Lb. farciminis*, *Lb. plantarum*, *Lc. garvieae*, *T. halophillus* and *W. thailandensis* were detected as the dominant bacteria species. The subdominant bacterial species in *kung-som* identified as *S. carnosus*. This provides useful information for the further development of bacterial starter culture in order to establish a more controllable *kung-som* process that gives a product with more consistent quality.

CHAPTER 3

PURIFICATION, CHARACTERIZATION AND SAFETY EVALUATION OF BACTERIOCIN PRODUCING *LACTOCOCCUS LACTIS* KTH0-1S ISOLATED FROM THAI TRADITIONAL FERMENTED SHRIMP (*KUNG-SOM*)

3.1 Abstract

Lactococcus lactis KTH0-1S originated from Thai traditional fermented shrimp (*kung-som*) had ability to produce heat-stable bacteriocin and showed broad spectrum activity against food-borne pathogenic and food spoilage bacteria. An inhibitory activity of bacteriocin retained active in various pHs and temperatures, however, it was inactivated by some protease enzymes. The addition of bacteriocin KTH0-1S to *Staphylococcus aureus* cultures resulted in viable cell counts decreased by 2 log CFU/ml exhibiting a bactericidal effect. Additionally, *S. aureus* growth was significantly decrease after 9 h co-cultivation with bacteriocin-producing strain. Bacteriocin KTH0-1S was purified by four steps of purification: ammonium sulfate precipitation, reverse phase cartridge (C_8 Sep-Pak), cation-exchange chromatography and RP-HPLC on C_8 column). Molecular mass of bacteriocin KTH0-1S was found to be 3.346 kDa after subjecting to mass spectrometry (MS/MS) analysis. Bacteriocin KTH0-1S was identified as nisin Z by using PCR amplification and sequencing. Nisin Z-producing *Lc. lactis* KTH0-1S was sensible towards most of studied antibiotics, non-biogenic amines production and the majority of tested virulence factors was absent, confirming the safety. Evidenced inhibitory effect of this strain, absence of virulence factors creates the possibility for its application as starter culture or protective culture to inhibit food-borne pathogens in the several fermented seafood products.

3.2 Introduction

Kung-som is a traditionally Thai fermented shrimp that is widely consumed in the south of Thailand. It made by mixing raw shrimp with sugar, salt and water and its fermentation solely relies on the natural lactic acid bacteria (LAB) originate in the main ingredient (Hwanhlem *et al*., 2010) that is responsible for the growth inhibition of undesirable microorganism during fermentation. Inhibitory effect is depending on LAB species, load of pathogenic bacteria and number of LAB in fermented foods (Normanno *et al*., 2005).

Staphylococcus aureus is the most important pathogen found in foods (dairy products, fishery products, meat products and fermented products) (Normanno *et al*., 2005). Several studies have been reported the food-poisoning in human caused by the consumption of foods contaminated with this bacterium and its enterotoxin (Kérouanton *et al*., 2007; Veras *et al*., 2008). Additionally, this bacterium did not require any particular nutrient or environmental condition for growth, it can grow at low water activity (a_w 0.86), wide temperature range (7-48ºC) and pH ranging (4.2-9.3) (Normanno *et al*., 2005). As fermented foods, the effectiveness of inhibition of *S. aureus* growth by organic acids generally depend on the concentration of their undissociated form. However, previous studies have reported the incidence of *S. aureus* in fermented products during fermentation period (Chokesajjawatee *et al*., 2009; Akkaya *et al*., 2014). *S. aureus* was tolerance in acid condition by maintaining of the intracellular pH through the releasing protons from cytoplasm (Charlier *et al*., 2009). Bacteriocin-producing LAB had potential inhibitory effect against *S. aureus* planktonic and biofilm cells (Okuda *et al*., 2013). Accordingly, it is quite interesting to select starter LAB was capable to produce a bacteriocin that efficiently inhibit *S. aureus* growth during fermentation process.

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria, some of which could be used as natural and safe antimicrobial agents for both food and medical industry (Lü *et al*., 2014; Ndlovu *et al*., 2015). The *in-situ* production of a bacteriocin by potential strains or starter cultures in fermentation processes require a bacteriocinogenic strain well adapted to the particular food environment, in which it will be used, that is able to grow under the food processing and/or storage conditions and which produces bacteriocin in sufficient amounts to inhibit the target bacteria. LAB originally isolated from certain food

products could be the best choice as starter cultures for the same products because they may be more competitive than LAB from other sources (Trias *et al.,* 2008).

European Food Safety Authority (EFSA) has been awarded *Lactococcus lactis* as Qualified Presumption of Safety (QPS) status (EFSA, 2011b). As previously reported, *Lc. lactis* and its bacteriocins were used as bio-preservative and bio-control in many kinds of food products including refrigerated meats and marine fishes (Sarika *et al*., 2012; Gao *et al*., 2014a) and dairy products (Cosentino *et al*., 2012). Although, *Lc. lactis* has been given the so-called GRAS and QPS status (EFSA, 2011b), previous studies have been reported that *Lc. lactis* was resistant to the clinically antibiotics and existent virulence genes in food-associated LAB (Flórez *et al*., 2007; Perin *et al*., 2014). These mobile genetic elements can potentially be transferred to human or animal bacterial flora, including pathogens (Van Reenen and Dicks, 2011; Huddleston, 2014). This bacterium have to characterization to ensure the inexistence of acquired risk factors so that these would be safe for human and animal consumption (Zycka-Krzesinska *et al*., 2015). Therefore, the aim of this study was to isolate, purify and characterize bacteriocin-producing LAB isolated from Thai traditional fermented shrimp (*kung-som*). Additionally, the effectiveness of *Lc. lactis* KTH0-1S to control *S. aureus* in co-cultivation and safety aspects have been studied for its application as starter culture and/or protective culture for the manufacture of a novel controlled fermentation process.

3.3 Materials and Methods

3.3.1 Bacterial isolates and media

The bacteriocin producer strain isolated from *kung-som* were kept in M17 (Merck, Germany) or de Man-Rogosa-Sharpe broth (MRS; Hi-media, India) supplemented with 30% (v/v) glycerol at -20°C. This strain was subcultured twice in M17 broth before experiments. All LAB as indicator strains were grown in MRS broth (pH 6.5) at 37°C for 24 h. Other indicator strains were grown in brain heart infusion broth (BHI; Hi-Media, India) at 37°C for 24 h. For routine analysis, the strains were activated by successive transfer in the respective media at 37°C for LAB and reference strains before use (Table 7).

3.3.2 Isolation and screening of bacteriocin-producing LAB strains

The *kung-som* was made according to the traditional recipe and the fermentation mixtures were transferred to glass jar covered with polyethylene film and incubated at 30°C for 21 days. During fermentation, the aliquots were taken from each jar every 2 days. Samples (25 g) were added to 225 ml of sterile peptone water (0.1% peptone, w/v) and homogenized using a stomacher machine (Seward, England), and serial ten-fold aliquot dilution was prepared. Then, 0.1 ml of each dilution was spread onto MRS (for lactobacilli) and M17 agar (for cocci) and also incubated for 24-48 h at 30°C. The bacterial colonies on MRS and M17 plates were screened primarily for antibacterial activities against *S. aureus* DMST 8840 or *B. cereus* DMST 5540 using an overlaid method under aerobic condition (Ko and Ahn, 2000). Inhibition zone surrounded colonies were selected and re-streaked 2-3 times on MRS or M17 agar plates in order to obtain pure cultures. The isolates exhibiting Gram-positive, catalase negative and non-motile cells were selected for testing the ability to produce an antibacterial substance against *S. aureus* DMST 8840 and *B. cereus* DMST 5540 by using two methods: agar spot test and well diffusion methods (García *et al*., 2004), consecutively. The cell free supernatant (CFS) of strain showing inhibition zone against indicator strain from well diffusion method was obtained by centrifugation (5424R, Eppendorf, Germany) at 6,800×*g* for 10 min at 4°C. The CFS was neutralized to pH 6.5 by adding 6 N NaOH to exclude the inhibitory effects of organic acids and then heated at 95°C for 10 min to kill the vegetative cells. The antibacterial activity of neutralized CFS (NCFS) was determined by well diffusion assay to confirm the bacteriocin activity.

3.3.3 Identification of bacteriocin-producing strain

The bacteriocin-producing strain was identified by 16S rDNA analysis. The total DNA was extracted from overnight culture of *Lc. lactis* KTH0-1S using the commercial kit (DNA extraction kit, Geneaid, Taiwan) following manufacture's recommendation. The 16S rDNA was amplified by PCR using the universal primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACT T-3′). PCR reaction was performed in 50 µl volume containing 5 µl of 2 µM of each primer, 25 µl of PCR Master Mix (GeNei^{TM}, Merck, Germany), 14 µl DNase free water and 1 µl of template DNA. PCR condition was performed with an initial denaturing at 95°C for 5 min, followed by 25 cycles at 95°C for 1 min, at 55°C for 1 min, and at 72°C

for 1 min with final extension at 72°C for 10 min. A 1484-bp fragment of the 16S rDNA was purified using HiYield Gel/PCR Fragments Extraction Kit (RBC, Taiwan) according to the manufacturer protocol. The DNA fragment was sent to 1st Base company (Kuala Lumpur, Malaysia) for sequencing.

3.3.4 Growth dynamics and bacteriocin production

The time course of growth and bacteriocin production was studied as follows: 1% (v/v) of an overnight culture of *Lc. lactis* KTH0-1S was inoculated 1% (v/v) into 500 ml of M17 broth and then incubated at 30°C under non-regulated pH condition. At 3 h intervals for 48 h, 10 ml of samples were collected from cultured broth to monitor the pH and the growth of cells by measuring the turbidity (600 nm). Antibacterial activity was assayed by well diffusion method in section 3.3.5.

3.3.5 Bacteriocin activity assay

The antibacterial activity of NCFS and partially purified bacteriocin was determined by well diffusion method (Schillinger and Lücke, 1989) with some modification. Wells were cut in MRS soft agar (1%, w/v) seeded with about 10^6 CFU/ml of an overnight culture of *Pediococcus pentosaceus* DMST 18752 and then mixed. The mixture was poured into a sterile Petri dish, and after setting agar, wells of 6 mm diameter were cut in the plate. Two-fold serial dilution of NCFS and partially purified bacteriocin (PPB) was made in sterile distilled water, and aliquots of 50 µl from each dilution were poured into the wells. The plates were left for 30 min in room temperature under the sterile condition before incubating at 37°C for 24 h. The bacteriocin activity was expressed in arbitrary units per milliliter by serial two-fold dilution method. Thus, an arbitrary unit (AU) was defined as the reciprocal of the highest two-fold serial dilution (2^n) that gave a minimal visible inhibition zone against indicator strain as follow: AU/ml = (2^n × 1000 µl)/50 µl

3.3.6 Inhibitory spectrum

The inhibitory spectrum of bacteriocin-producing strain was assessed against indicator strains using agar well diffusion assay. Accordingly, an aliquot of 50 µl of NCFS was poured into the well and set on soft agar plates previously inoculated with tested indicator strains (10^6 CFU/ml) and incubated at an optimal temperature. Antibacterial activity measured by the halo zone in the bacterial lawn around the wells.

3.3.7 Characterization of partially purified bacteriocin

The partially purified bacteriocin (PPB) was obtained from ammonium sulfate precipitation and dialyzed with Spectra/Por® dialysis tube (1 kDa cut off; Spectrum, CA, USA) in 20 mM phosphate buffer (PBS; pH 6.5) and then freeze-dried. The dried PPB was resuspended in optimal buffer for testing with catalase and proteases (α-chymotrypsin, pepsin, proteinase K and trypsin,) in a final concentration of 1 mg/ml for 3 h at 37°C. The thermostability of bacteriocin activity was determined by heating PPB at 30-100°C for 3 h and 121°C for 15 min. The pH stability of bacteriocin was assayed in 50 mM of different buffers at pH range 2.0-11.0 at 30°C. The bacteriocin activity was evaluated by measuring the residual activity after incubation at various pH for 24 h. Different buffers used were mentioned above and readjusted to pH 6.5 before bacteriocin activity determination. Stability of bacteriocin during storage was also studied. Aliquots of 400 µl of PPB in acetate buffer (pH 4.0) were taken into sterile microcentrifuge tubes and stored for 30 days at -20°C, 4°C, 30°C and 37°C. At different time intervals (3, 7, 15 and 30 days), samples were taken from the stored material to measure antagonistic activity against indicator strain using a well diffusion assay as previously described.

3.3.8 Mode of action

To investigate mode of action of the antibacterial peptide producing *Lc. lactis* KTH0-1S, the lyophilized PPB KTH0-1S from ammonium sulfate precipitation was added to a culture of *S. aureus* DMST 8840 in BHI broth in an early (4 h) exponential phase (at final concentration of 640 AU/ml). The treatment without bacteriocin was used as control. Viable cell counts (CFU/ml) were determined by plating on BHI plates and changes in the cell growth were observed at turbidity (OD_{600nm}) (Todorov and Dicks, 2005).

3.3.9 Inhibitory effect in co-cultivation

The effectiveness of bacteriocinogenic *Lc. lactis* KTH0-1S on the growth of *S. aureus* was further studied in a co-cultivation as described by Hwanhlem *et al.* (2010). Each 4 ml of M17 broth and BHI broth containing L-cysteine (0.5 mg/ml) was placed into test tube. One milliliter of active culture of *Lc. lactis* KTH0-1S (10^7 CFU/ml) and 1 ml of *S. aureus* DMST 8840 (10^7 CFU/ml) were inoculated in test tube and then overlaid with liquid paraffin. Control cultures were obtained by inoculating separately 9 ml of tested medium with either 1 ml of *Lc. lactis* KTH0-1S (10^7 CFU/ml)

or 1 ml (10^7 CFU/ml) of *S. aureus* DMST 8840 and cultivated at 37°C for 48 h. Growth of *Lc. lactis* KTH0-1S and *S. aureus* DMST 8840 was monitored every 3 h by plating the viable cells on a selective agar suitable for each species. Values of the pH and bacteriocin activity were also monitored continuously during incubation.

3.3.10 Purification of bacteriocin

Lc. lactis KTH0-1S was inoculated into 1 liter of M17 broth and incubated at 30°C for 24 h without shaking. After cells were removed by centrifugation, CFS was saturated with 70% ammonium sulfate at 4°C and stirred for overnight to produce a crude bacteriocin preparation. The pellet was harvested by centrifugation (Sorvall Legend XTR, Thermo Scientific, MA, USA) at 10,300×*g* for 20 min at 4°C and then resuspended in 20 mM phosphate buffer (PBS; pH 6.5). The crude bacteriocin was subjected to a reversed phase (RP) C_8 silica cartridge (5 g/20 ml; Waters, MA, USA) and then eluted in 2 fractions with different acetonitrile (ACN)-MilliQ water mixtures (20 % and 80 % ACN). The acetonitrile was then eliminated using a Speed-Vac concentrator (SC110A, Savant, NY, USA). After acetonitrile elimination, the collected fractions were resuspended in 20 mM PBS (pH 6.5) before determining bacteriocin activity. The active fractions were further purified by using a SP-Sepharose Fast flow column (GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM PBS (flow rate 2.0 ml/min). The column was washed with the same buffer and the absorbed proteins were eluted with 1.0 M NaCl. The concentrated sample was purified by RP-HPLC (Waters, MA, USA) with Symmetry® RP-C_8 column (5 μm, 7.6×100 mm, Waters, MA, USA). The elution was done with solvent A (95% MilliQ water, 5% solvent B, 0.05% trifluoroacetic acid (TFA) and solvent B (80% ACN, 20% isopropanol (IsOH), 0.03% TFA) with a linear gradient from 20% to 35% solvent B in 27 min (flow rate 2.4 ml/min). The absorbance of peptide fractions was recorded between 210 and 300 nm spectrophotometrically and the fractions were collected manually. The active fractions of each step of purification were adjusted to pH 6.5 before determining antimicrobial activity using well diffusion assay (*P. pentosaceus* DMST 18752 was used as an indicator) and protein profiling determination by Tricine SDS-PAGE (Schägger and von Jagow, 1987) with some modifications in section 3.3.11. The purified bacteriocin was analyzed for its molecular mass by using mass spectrometry (MS/MS) analysis (INRA, Paris, France).

3.3.11 Protein profiling determination using Tricine SDS-PAGE

The active fractions of each step of purification were determined protein profiling by Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine SDS-PAGE) (Schägger and von Jagow, 1987) using a 16.5% acrylamide slab running gel on a Mini-Protean® II electrophoresis cell (Bio-Rad, CA, USA). An ultra-low range molecular weight marker (1.06-26 kDa; Sigma-Aldrich, MO, USA) was used as a protein standard. Gel electrophoresis was carried out at constant current (10 mA in stacking gel and 22 mA in running gel) using cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% (w/v) SDS, pH 8.3) and anode buffer (0.2 M Tris, pH 8.9). After electrophoresis, one half of the gel containing the samples and marker was fixed for 30 min in 12.5% (w/v) trichloroacetic acid (TCA), then rinsed with water and stained with Coomassie blue G-250 (Sigma-Aldrich) for 2 h and destained in destaining solution (methanol/glacial acetic acid/water, 5/1/4, v/v/v) at room temperature by shaking. The second half of the gel was washed with sterile distilled water for 3 h by changing the sterile distilled water every 30 min. Then, the gel was overlaid with soft MRS seeding *P. pentosaceus* DMST 18752 and incubated overnight at 37°C. The position of bacteriocin was visualized by inhibition zone around the active protein band.

3.3.10 Bacteriocin encoding gene detection

The detection of structural gene encoding nisin in *Lc. lactis* KTH01S was performed by PCR with specific primer as shown in Table 6. Fifty microliter of PCR reaction volume contained 25 µl of PCR master Mix (Merck, Germany), 5 µl of 2 µM of each primer, 1 µl of DNA template and then adjusted to 50 µl with DNase free water. The amplification reactions were performed in a thermal cycle using the following cycling program: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension step was at 72°C for 10 min. The amplicons were analyzed by electrophoresis in a 1.5% (w/v) agarose gel containing 1×SYBR Safe (Invitrogen™, CA, USA) in 0.5×Tris-Acetate-EDTA (TAE; pH 8.0) buffer and observation using gel documentation (UVItec, England). The purified PCR fragment was sent to 1st Base company (Kuala Lumpur, Malaysia) for sequencing.

3.3.13 Safety evaluation

3.3.11.1 Tests of hemolytic activities

The test of hemolytic activities is a relevant for safety evaluation of the LAB. *Lc. lactis* KTH0-1S was cultivated first in M17 broth at 30°C for overnight. This strain was streaked in triplicates on TSA supplemented with 5% (v/v) sterile defibrinated human blood (Songklanakarin Hospital, Songkhla, Thailand) and then the plate was incubated at 30°C for 24 and 48 h. Quality control of the blood agar plates was performed with the test strains *S. aureus* MRSA3 and *S. aureus* MSSA4 as positive controls and *Lb. plantarum* JCM 1149 as negative control. Hemolytic activity was examined by the appearance of clear zone (β-hemolysis), greenish zone (α-hemolysis) or absence of any zone (γ-hemolysis) around the colonies on blood agar.

3.3.11.2 Antibiotic susceptibility testing

The antibiotic sensitivity of *Lc. lactis* KTH0-1S was performed by the broth microdilution assay (Klare *et al*., 2005) that was used to evaluate minimal inhibitory concentrations (MICs) for each antibiotic (ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, kanamycin, streptomycin, tetracycline and vancomycin; Sigma-Aldrich). A two-fold serial dilution of each antibiotic was prepared in a 96-well microtiter plate by using the mixture of 90% (w/v) ISO-SENSITEST™ medium (Oxoid, England) and 10% (w/v) M17 medium (ISM17 broth) as diluents (final volume was 100 µl). An overnight culture of 100 µl of *Lc. lactis* KTH0-1S was inoculated into wells (a final concentration was 10^5 CFU/ml). After 24 h incubation at 30°C, a growth inhibition of LAB was determined by optical density at 600 nm using a microplate reader (BioTek®, VT, USA). A positive control was obtained by the use of 100 µl of ISM17 broth and 100 µl of appropriate dilution of culture broth, and bacterial free well was used as a negative control containing 200 µl of ISM17 broth. $MICs_{90}$ values of each antibiotic were evaluated as the lowest concentration of the sample tested that could restrict bacterial growth at an inhibition rate higher than 90%. Interpretation for susceptibility status was based on EFSA (2012). All the tests were repeated at least twice. In duplicate experiments, the differences of MIC for independent sample never exceeded 1 order of dilution.

Table 6 Primer sets used for the detection of the nisin gene, virulence genes and amino acid decarboxylase genes in *Lc. lactis* KTH0-1S.

Target genes	Sequence (5´ to 3´)	T_m (°C)	Product size (bp)	References
nisZ	Nis-F: CCGGAATTCATAAGGAGGCACTCAAAATG Nis-R: CGGGGTACCTACTATCCTTTGATTTGGTT	55	227	Noonpakdee *et al.* (2003)
ace	ACE-F: GAATTGAGCAAAAGTTCAATCG ACE-R: GTCTGTCTTTTCACTTGTTTC	56	1008	Omar *et al*. (2004)
asa1	ASA11: GCACGCTATTACGAACTATGA ASA12: TAAGAAAGAACATCACCACGA	56	375	Vankerckhoven *et al*. (2004)
cylA	CYT I: ACTCGGGGATTGATAGGC CYT IIb: GCTGCTAAAGCTGCGCTT	58	688	Vankerckhoven *et al*. (2004)
cylB	cylB1: AAGTACACTAGTAGAACTAAGGGA cylB2: ACAGTGAACGATATAACTCGCTATT	55	2020	Semedo *et al*. (2003)
efaAfs	TE5: GACAGACCCTCACGAATA TE6: AGTTCATCATGCTGTAGTA	54	735	Eaton and Gasson (2001)
esp	ESP14F: AGATTTCATCTTTGATTCTTGG ESP12R: AATTGATTCTTTAGCATCTGG	56	510	Vankerckhoven *et al*. (2004)
gelE	TE9: ACCCCGTATCATTGGTTT TE10: ACGCATTGCTTTTCCATC	52	419	Eaton and Gasson (2001)
hdc	HIS-f: GGNATNGTNWSNTAYGAMGNGCNGA HIS-r: ATNGCDATNGCNSWCCANACNCCRTA	53	372	de las Rivas *et al*. (2006)
odc	PUT1-f: TWYMAYGCNGAYAARACNTAYYYTGT PUT1-r: ACRCANAGNACNCCNGGNGGRTANGG	53	1440	de las Rivas *et al*. (2006)
ldc	CAD2-f: CAYRTNCCNGGNCAYAA CAD2-r: GGDATNCCNGGNGGRTA	53	1185	de las Rivas *et al*. (2006)
tdc	TDC-f: TGGYTNGTNCCNCARACNAARCAYTA TDC-r: ACRTARTCNACCATRTTRAARTCNGG	53	825	de las Rivas *et al*. (2006)

3.3.11.3 Virulence factors detection

In order to assess the safety of *Lc. lactis* subsp. *lactis* KTH0-1S for use as starter culture in *kung-som* production or for production of other fermented foods, the presence of genes encoding virulence was checked by PCR according to Creti *et al*. (2004) and by using the primers described in Table 6. The target genes were *ace* (adhesin of collagen protein), *asa1* (aggregation substance), *cylA/B* (cytolysins), *efaAfs* (cell wall adhesin) and *esp* (enterococcal surface protein). Fifty microliter of PCR reaction mixtures contained 5 µl of 2 µM of each primer, 25 µl of PCR Master Mix, 1 µl of DNA template. The total volume was adjusted to 50 µl with DNase free water. The amplifications were performed in a Touchgene gradient thermocycler (Techne, England) as follows: 94°C for 3 min (first denaturation step), followed by 36 cycles of 94°C for 1 min, annealing step for 1 min at 56°C (*asa1*, *ace* and *esp*), 58°C (*cylA*), 52°C (*cylB* and *gelE*) and 54°C (*efaAfs*) (Table 6), and extension at 72°C for 1 min with a final extension step at 72°C for 5 min. PCR fragments were analyzed by electrophoresis for 45 min at 100 V in a 1.5% (w/v) agarose gel containing 1×SYBR Safe in 0.5×TAE buffer pH 8.0 and observation using UV trans-illuminator with gel documentation.

3.3.11.4 Biogenic amines production

Thin-layer chromatography (TLC) was used for the detection of the biogenic amines produced (Garcia-Moruno *et al*., 2005). *Lc. lactis* KTH0-1S was cultivated in M17 broth overnight and then this strain was inoculated into 10 ml of M17 broth supplemented 0.5% (w/v) of corresponding amino acids precursor originated from Merck (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany): L-histidine monohydrochloride; L-tyrosine disodium salt; L-lysine monohydrochloride; and L-ornithine hydrochloride. After incubation at 30°C for 7 days, CFS was collected. As positive control, biogenic amines stock standard solution (histamine, cadaverine, putrescine and tyramine; Sigma-Aldrich) were made by preparing a 2% (w/v) (5% w/v in the case of histamine) in 40% ethanol. Amines were converted into their fluorescent dansyl derivatives according to the method described by Landeta *et al*. (2007). The derivative samples were spotted onto TLC plate (silica gel 60 F_{254}, Merck). The dansylated compounds were separated by chloroform/di-ethyl ester/tri-ethylamine (3/2/1, v/v/v) and then the TLC plate was sprayed with isopropanol/tri-ethanolamine (4/1, v/v). The fluorescent dansyl derivative on TLC plate was visualized under UV light exposure.

The presence of gene encoding the decarboxylase enzymes involved in biogenic amines production was detected by PCR using specific primers previously described (de las Rivas *et al*., 2006) (Table 6). The PCR amplification was performed in 25 µl volume containing 2.5 µl of 2 µM of each primer, 12.5 µl of PCR Master Mix, 2 µl of DNA template and volume was adjusted with DNase free water. PCR program was applied to an initial denaturing at 95°C for 15 min, followed by 36 cycles at 95°C for 1 min, at 53°C for 1 min, and at 72°C for 3 min. The final extension was at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel containing 1×SYBR Safe and then photographed by gel documentation.

3.4 Results and Discussion

3.4.1 Screening of bacteriocin-producing LAB isolates and strain identification

This fermented shrimp (*kung-som*) product is typically produced on a small scale or at household levels and is sometime associated with problems such as short shelf-life and poor hygiene. The use of starter culture is not a common practice, what results in product failure and inconsistency. The potential of starter cultures to

produce antibacterial compounds such as bacteriocin has attracted great attention since they can be used to prevent food spoilage and to inhibit the growth of food pathogens. The aim of the present study was to screen bacteriocin produced by LAB isolated from *kung-som*. It was found that 315 isolates showed inhibitory effect against *S. aureus* DMST 8840 or *B. cereus* DMST 5540 using overlaid method. However, only 91 isolates exhibited antibacterial activity toward *S. aureus* DMST 8840 and *B. cereus* DMST 5540, representing the isolation frequency of 28.8% in the secondary screening using agar spot. In addition, they were identified as LAB using the criteria of being Gram-positive, non-motile and catalase negative. However, only isolate KTH0-1S showed inhibitory activity against both indicator strains using agar well diffusion assay. CFS of KTH0-1S strain exhibited inhibitory efficiency toward *S. aureus* DMST 8840 and *B. cereus* DMST 5540 after pH adjustment to 6.5. An inhibitory activity NCFS of KTH0-1S was lost after treating with α-chymotrypsin and proteinase K, demonstrating the proteinaceous nature of antibacterial compound. This finding can conclude that the succession rate to obtain the bacteriocin-producing LAB was 0.32%.

By comparing the full length of 16S rDNA sequence, the strain KTH0-1S was identified as *Lc. lactis* subsp. *lactis* (99% identity). The nucleotide sequence was deposited in DDBJ/EMBL/GenBank databases as an accession number AB985677. Bacteriocin producing *Lc. lactis* have been reported to be isolated from fermented foods such as cheese products, kimchi and sausage (Choi *et al*., 2000; Ko and Ahn, 2000; Dal Bello *et al*., 2012). However, there is no report so far on the isolation of bacteriocin-producing *Lc. lactis* from Thai fermented shrimp (*kung-som*). This strain should be used as a starter culture or protective culture in *kung-som* production to improve the safety of product.

3.4.2 Inhibitory spectrum

Generally, bacteriocins have antagonistic activities against closely related species (narrow spectrum) or across genera (broad-spectrum). The antimicrobial spectra of bacteriocins produced by different species of lactococci vary greatly. In the present study, the NCFS of *Lc. lactis* KTH0-1S exhibited an antibacterial effect on the important food-spoilage and food-borne pathogens including, *Brochothrix thermosphacta*, *B. cereus*, *Lactobacillus sakei*, *Leuconostoc pseudomesenteroides*, vancomycin-resistant enterococci strains, *Listeria monocytogenes*, *Lis. innocua*, *Lis. ivanovii*, *S. aureus*, methicillin-resistance *S. aureus* strains and coagulase-negative

staphylococci strains (halotolerant bacteria) (Table 7), especially this strain was able to inhibit to all tested vancomycin-resistance enterococci (VRE) and methicillin-resistance *S. aureus* (MRSA). Mitra *et al*. (2007) reported that *Lc. lactis* CM1 produced a bacteriocin exhibiting a broad range spectrum of antagonistic activity, similar to bacteriocin produced by *Lc. lactis* 19.3, which is highly active against Gram-positive bacteria including *B. cereus*, *Lb. delbrueckii*, *Lb. sakei*, and *Lis. monocytogenes* (Grosu-Tudor *et al*., 2014). However, NCFS could not inhibit all tested Gram-negative bacteria due to the outer membrane barrier of Gram-negative bacteria such as *E. coli* and *Salmonella* sp., which prohibits bacteriocin from accessing the cytoplasmic membrane (Kuwano *et al*., 2005).

Additionally, several papers have been published on the prevalence of the antibiotic resistance of staphylococci and enterococci in many kinds of fermented foods (Valenzuela *et al*., 2009; Marty *et al*., 2012; Lee *et al*., 2015). Thus, bacteriocin-producing LAB was alternative preservative agent to inhibit drug-resistance bacteria in accordance with Okuda *et al*. (2013) reported that bacteriocin (nisin) had potential the inhibition of MRSA more than vancomycin. Moreover, few studies reported that bacteriocin derived from LAB presented antagonistic activity against both VRE and MRSA (Brumfitt, 2002; Aunpad and Na-Bangchang, 2007). The result obtained from the present study suggests the possibility to be used *Lc. lactis* KTH0-1S as protective culture for suppression or controlling drug-resistant microorganism.

In addition, bacteriocin KTH0-1S exhibited the largest inhibitory zone against *Br. thermosphacta* and *P. pentosaceus* DMST 18752. It can be to be indicated that both strains were sensitive to bacteriocin KTH0-1S. From the result obtained *P. pentosaceus* DMST 18752 was chosen as sensitive indicator to determine the effect of environmental parameters on inhibitory activity of bacteriocin KTH0-1S for further experiments.

Table 7 Spectrum of activity of *Lc. lactis* KTH0-1S isolated from *kung-som*.

Test microorganisms	Sources	Temperature /Incubation time	Radius of inhibition zones (mm)
B. cereus DMST 5540	DMST[a]	37°C/24 h	5.0
Br. thermosphacta DMSZ 20171^T	ONIRIS[b]	25°C/24 h	12.0
Ent. faecium 139*	CU[c]	37°C/24 h	6.0
Ent. faecium 174*	CU	37°C/24 h	4.5
Ent. faecium 348*	CU	37°C/24 h	6.0
Ent. faecium KT4S13	Our strain collection	37°C/24 h	6.0
Ent. faecalis VanB*	CU	37°C/24 h	5.0
Ent. gallinarum VanC*	CU	37°C/24 h	6.5
E. coli DMST 4212	DMST	37°C/24 h	-
Lb. plantarum D6SM3	Our strain collection	37°C/24 h	7.0
Lb. sakei subsp. *sakei* JCM 1157	JCM[d]	37°C/24 h	3.0
Leu. pseudomesenteroides D2KS11	Our strain collection	37°C/24 h	5.0
Lis. innocua P	INRA[e]	37°C/24 h	3.0
Lis. ivanovii CIP 78.42T	INRA	37°C/24 h	5.0
Lis. monocytogenes DMST 17303	DMST	37°C/24 h	3.0
P. pentosaceus DMST 18752	DMST	37°C/24 h	12.0
Sal. Enteritis DMST 15676	DMST	37°C/24 h	-
Sal. Typhimurium DMST 16809	DMST	37°C/24 h	-
S. aureus DMST 8840	DMST	37°C/24 h	4.0
S. aureus S01†	Songklanagarind Hospital[f]	37°C/24 h	4.0
S. aureus S03†	Songklanagarind Hospital	37°C/24 h	5.0
S. aureus S05†	Songklanagarind Hospital	37°C/24 h	5.0
S. condimenti K16S2	Our strain collection	30°C/24 h	5.5
S. piscifermentans K2S12	Our strain collection	30°C/24 h	5.0
S. saprophyticus K10S6	Our strain collection	30°C/24 h	5.5
S. xylosus K2S6	Our strain collection	30°C/24 h	5.0

[a]DMST: Department of Medical Sciences Thailand Culture Collection, Bangkok, Thailand.
[b]ONIRIS: Ecole Nationale Nantes Atlantique Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes, France.
[c]The WHO Global Salm-Surv Regional Centre of Excellence: South-East Asia and Western Pacific, Faculty of Veterinary Science, Chulalongkorn University, Thailand.
[d]JCM: Japan Collection of Microorganism, Japan.
[e]INRA: Institut National de la Recherche Agronomique, Nantes, France.
[f]Songklanagarind Hospital, Prince of Songkla University, Thailand.
-: no inhibition.
* Vancomycin-resistant *Enterococcus*, † Methicillin-resistance *Staphylococcus*.

3.4.3 Growth dynamics and bacteriocin production

Bacteriocin produced by *Lc. lactis* KTH0-1S gave the highest level of activity against *P. pentosaceus* DMST 18752 when cultivated in M17 broth at 30°C. *Lc. lactis* KTH0-1S started to produce bacteriocin at 6 h during early logarithmic growth phase. Bacteriocin production reached the maximum (640 AU/ml) after 15 h of incubation, corresponding to the beginning of the stationary phase of growth and the level of inhibition thereafter remained constant (Fig. 15). Similar results have been reported for bacteriocin produced by *Lc. lactis* CM1 (Mitra *et al*., 2007) and *Lc. lactis* MK02R (Kruger *et al*., 2013). The bacteriocin activity was found at the early log phase of growth, increased in parallel to the growth of the bacterium and reached its maximum at early stationary phase. In the same way, the pH strongly decreased from the initial value of 7.2 to around 6.0 in 12 h during the logarithmic growth and slightly decreased towards the end of incubation (pH 5.2 at 24 h). The decrease of pH during the growth of LAB was observed usually, mainly due to the production of lactic acid, acetic acid, propionic acid and carbon dioxide (García *et al*., 2004).

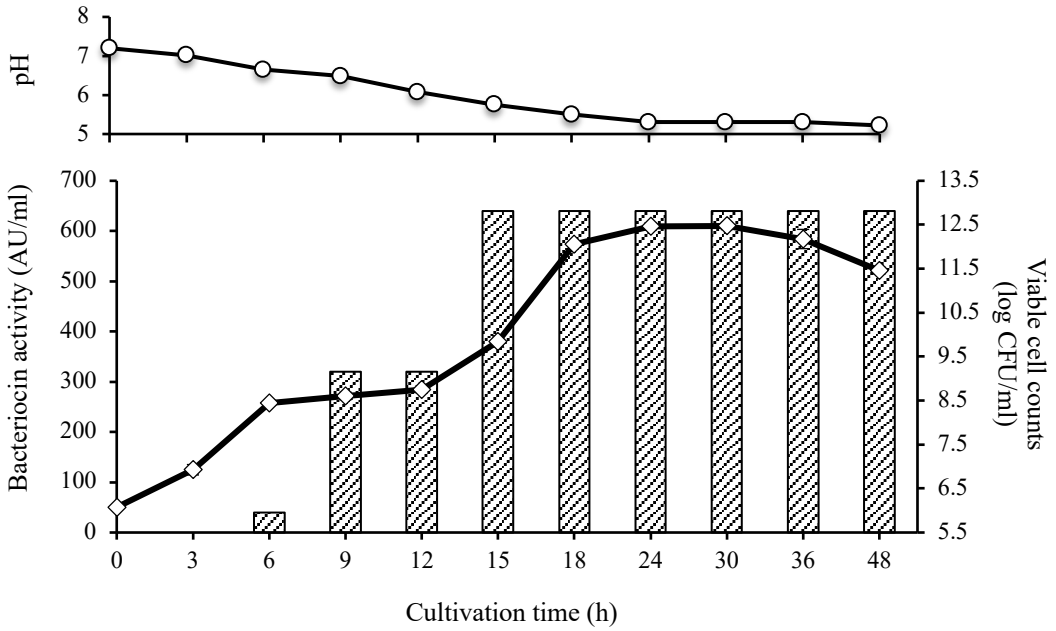

Figure 15 Antibacterial activity (bars) of *Lc. lactis* KTH0-1S against *P. pentosaceus* DMST 18752 observed during growth in M17 medium (expressed in AU/ml). Viable cell counts of *Lc. lactis* KTH0-1S (◇) was cultivated in M17 broth incubated at 30 °C for 24 h and changes of pH value (○) during cultivation. Bars represent the standard deviation from three determinations.

3.4.4 Mode of action of bacteriocin KTH0-1S and inhibitory effect of co-cultivation

S. aureus is considered to be the most common pathogenic bacteria causing food-poisoning outbreaks (Kérouanton *et al*., 2007; Veras *et al*., 2008). Previous studies reported that enterotoxigenic *S. aureus* was frequently found in several foods: dairy products, fishery products, meat products and fermented products (Kérouanton *et al*., 2007; Chokesajjawatee *et al*., 2009; Wu and Su, 2014), making the contaminated food unsafe, even after cooking. Thus, this study determined the effectiveness of PPB KTH0-1S was against *S. aureus*. The addition of bacteriocin KTH0-1S to *S. aureus* DMST 8840 at the early exponential phase (4 h) resulted in a stop of A_{600nm} curve, which slightly increased till the end of the experiment (Fig. 16B). In the same time, the proportion of dead cells was significantly higher since viable cell counts decreased from 6.5±0.1 to 3.7±0.04 log CFU/ml within 2 h of incubation after addition of bacteriocin KTH0-1S (Fig. 16C) when compared to control treatment. After 24 h of incubation, *S. aureus* DMST 8840 was inhibited by bacteriocin KTH0-1S at 4.1 log CFU/ml, while control (without bacteriocin KTH0-1S) increased to 8.3 log CFU/ml (Fig. 16C). However, bacteriocin KTH0-1S could not completely kill *S. aureus* DMST 8840 cells since its concentration (final concentration 640 AU/ml) was equivalent to MIC value (Fig. 17). Our data indicate that bacteriocin KTH0-1S exhibited a bactericidal activity against *S. aureus* DMST 8840. The bactericidal action on sensitive cells involves the pores formation in the membrane. However, its action did not induce cell lysis. Serveral researches proposed that bacteriocin binds with Lipid II to induce the formation of large bacteriocin-Lipid II aggregates in the membrane of bacteria. This interaction inhibits peptidoglycan synthesis and forms highly specific pores, causing cell death due to loss of essential intracellular substances (Hancock and Rozek, 2002; Bauer and Dicks, 2005; Teixei *et al*., 2012).

In addition, the inhibitory effect of *Lc. lactis* KTH0-1S on the growth of *S. aureus* DMST 8840 was studied in a co-cultivation at 37°C. The growth of *S. aureus* in the presence of *Lc. lactis* KTH0-1S showed a significant drop ($p<0.05$) in its population after 12 h incubation. This drop led to a final population nearing 4.0 log CFU/ml after 24 h (Fig. 18A) compared to the pure cultures (*S. aureus*) that reached levels ≥9.0 log CFU/ml.

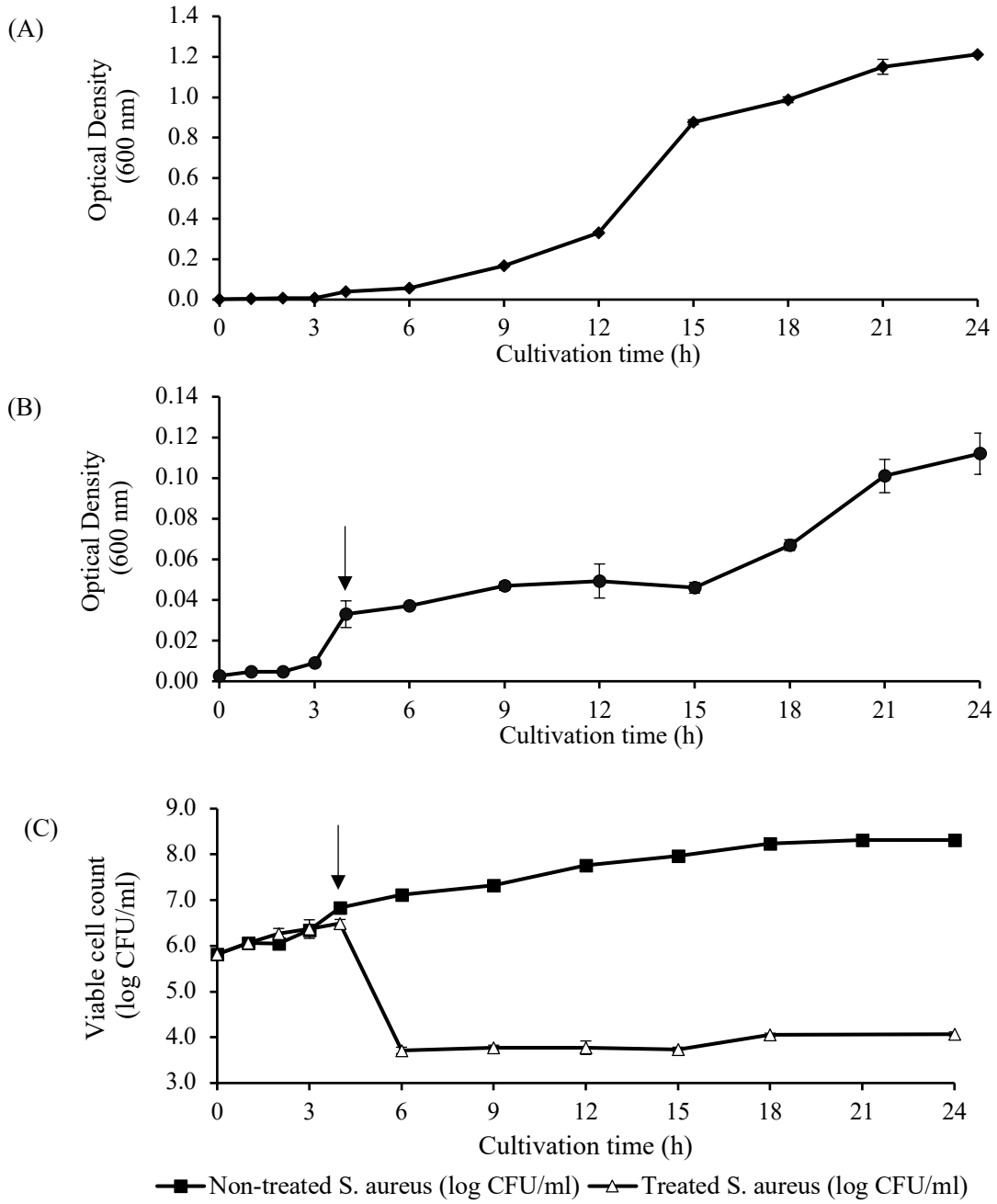

Figure 16 Mode of action of bacteriocin KTH0-1S produced by *Lc. lactis* KTH0-1S on the growth of *S. aureus* DMST 8840 (A) and (B): Optical density at 600 nm was observed in the absence (◆) and presence (●) of bacteriocin KTH0-1S. (C): Log CFU/ml viable count observed in the absence (■) and presence (△) of bacteriocin KTH0-1S. The arrow indicates the time of the addition of bacteriocin KTH0-1S. Bars represent the standard deviation from three determinations.

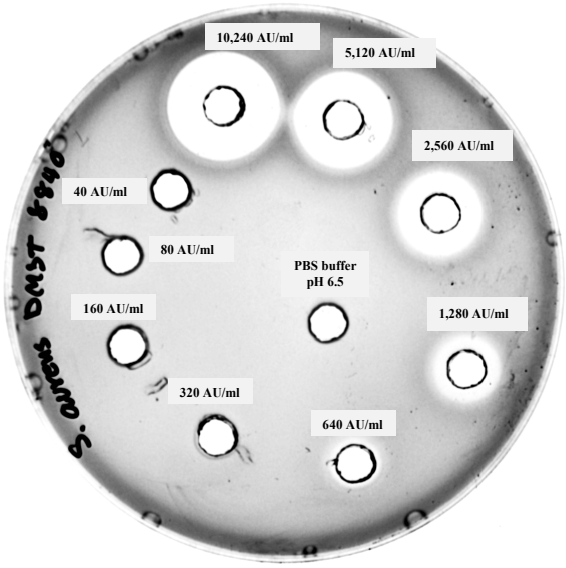

Figure 17 MIC of partially purified bacteriocin of *Lc. lactis* KTH0-1S against *S. aureus* DMST 8840 was determined by agar well diffusion assay.

In both pure and co-culture, a decrease of the pH was observed. The pH of the medium in co-cultivation treatment ranged from 5.3 to 5.5 (Fig. 18B), whereas in the pure culture (*S. aureus*), pH ranged between 6.0 and 6.2 after 24 h of cultivation (Fig. 18B). Accordingly, the inhibitory activity gradually increased during *Lc. lactis* KTH0-1S growth phases and reached a maximum at the late exponentially growth phase corresponding to the level of bacteriocin activity (Fig. 18C). This observation suggested that strain KTH0-1S exhibited competitive growth and produced the nisin Z during co-cultivation with *S. aureus*. Similarly, a previous report demonstrated the inhibitory capability of LAB on *S. aureus* growth in fermented foodstuffs caused by their metabolites involved in the inhibition phenomenon (acidification, bacteriocins production, H_2O_2 production) (Charlier *et al*., 2009). In addition, inter- and intra-species variabilities were the determinant for inhibition of *S. aureus* growth, in agreement with the result of Charlier *et al.* (2008) who reported that 93% *Lc. lactis* strains exerted strong inhibitory effect on *S. aureus* growth in milk. This high prevalence of the inhibition potential within *Lc. lactis* was homogeneous among LAB species, thus, the inhibitory capability of LAB species may be strain dependent (Charlier *et al*., 2009). Therefore, our results strongly suggest that the bacteriocin producing *Lc. lactis* KTH0-1S is suitable to be used as starter or protective culture for inhibition of *S. aureus* contamination during *kung-som* fermentation.

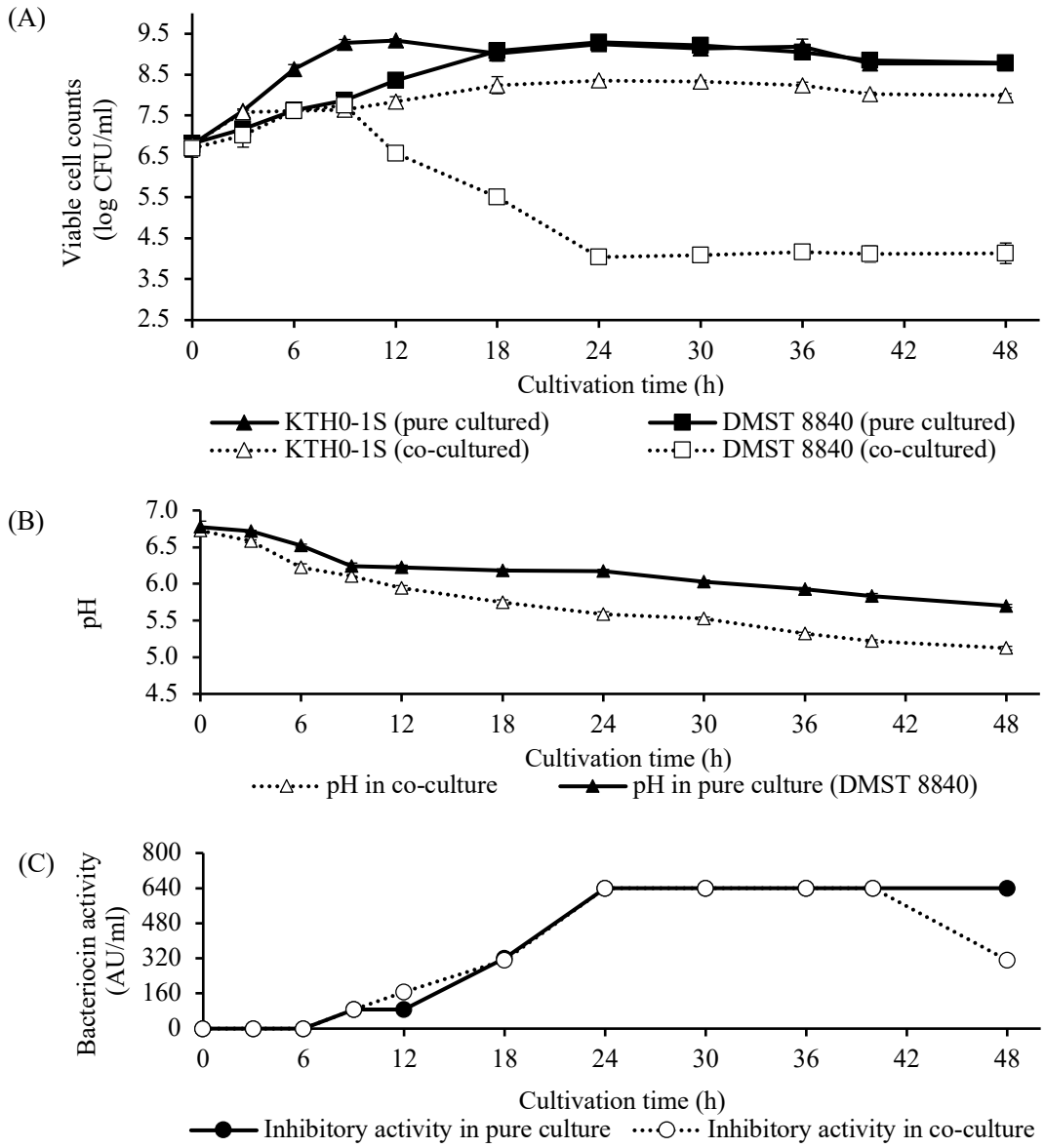

Figure 18 (A): Effect of *Lc. lactis* KTH0-1S on the growth of *S. aureus* DMST 8840 in co-cultivation, (▲) *Lc. lactis* KTH0-1S, (■) *S. aureus* DMST 8840, (△) *Lc. lactis* KTH0-1S in co-culture experiment, (□) *S. aureus* DMST 8840 in co-culture experiment. (B): The pH value observed during co-cultivation, △ co-culture *Lc. lactis* KTH0-1S with *S. aureus* DMST 8840, ▲ mono-culture *S. aureus* DMST 8840. (C): Bacteriocin activity was expressed during co-cultivation experiment, (●) mono-culture *Lc. lactis* KTH0-1S, (○) co-culture *Lc. lactis* KTH0-1S with *S. aureus* DMST 8840. Bars represent the standard deviation from three determinations.

3.4.5 Effects of enzymatic and physicochemical treatments on bacteriocin activity

The inhibitory activity of PPB produced by *Lc. lactis* KTH0-1S was treated with various proteolytic enzymes, at different temperatures and pH condition are shown in Table 8. The treatment with catalase did not affect activity against the target strain indicating that the inhibition recorded is not caused by hydrogen peroxide. The inhibitory activities completely after treatment with proteinase K, what agrees with reports of Noonpakdee *et al*. (2003) and Hwanhlem *et al*. (2013). The activity of PPB toward tested strain was reduced in some extent with pepsin and α-chymotrypsin (Table 8), whereas treatment with trypsin did not result in any changes of antibacterial activity. Letters and Tuncer (2010) and Pasteris *et al*. (2014) observed differences in bacteriocin activity after treatment with α-chymotrypsin and proteinase K. The different inhibitory activities might be due to post-translational modification process of peptide synthesized by LAB modifying bacteriocin structure (Nes and Johnsborg, 2004). The results obtained from Table 7 indicated that bacteriocin showed broad-spectrum antagonistic activity that might be inhibited the LAB as microflora in intestinal tract. Previous study found that bacteriocin Abp118 produced by *L. salivarius* UCC118 was significant change the porcine intestinal microbiota (Riboulet-Bisson *et al*., 2012). In another study reported that bacteriocins (enterocins P, Q and L50, garvicin ML, pediocin PA-1and sakacin A) unaffected the overall structure of murine intestinal microbiota. However, treatments with bacteriocins were changes of some bacterial phylotypes on gut microbiota in healthy mice, especially, the treatments with broad spectrum bacteriocins (enterocins Q and L50 and garvicin ML) could be inhibited potentially problematic strains such as *Clostridium*, Enterococcaceae and *Staphylococcus* (Umu *et al*., 2016).

The bacteriocin KTH0-1S was heat-stable (up to 100 °C) and had high activity in a wide pH range (2-9). However, the activity was lost at pH >10. It was acid-stable and thermotolerant. These characteristics were similar to other bacteriocins previously reported (Bromberg *et al*., 2005; de Arauz *et al*., 2009). Bacteriocin usually maintains its inhibitory effect at various pH values and exhibits higher inhibitory activity at low pH. When the pH is lowered, the solubility and stability of bacteriocin tend to increase drastically or probably due to the better penetration of organic acids into the microbial cell wall. In contrast, neutral and extreme alkaline pH conditions could result in protein aggregation or instability of protein (Perin *et al*., 2013).

Furthermore, bacteriocin KTH0-1S maintained full activity (1,280 AU/ml) after 30 days of storage at -20°C and 4°C. The inhibitory activity of PPB reduced by 50% (640 AU/ml) after storage at 30°C and 37°C for 30 days (Table 8). Accordingly, we can conclude that bacteriocin kept at low temperature can maintain inhibitory activity better than those observed after long storage periods at high temperature.

Table 8 Antimicrobial activity of the partially purified bacteriocin of *Lc. lactis* KTH0-1S after treatment with various enzymes, pHs, temperatures and storage time.

Treatments	Residual bacteriocin activity (AU/ml)
Enzymes	
Control (without enzyme)	640
Catalase	640
Trypsin	640
Pepsin	80
α-Chymotrypsin	160
Proteinase K	0
pH (24 h)	
Control	640
2	640
3	640
4	640
5	640
6	640
7	640
8	640
9	640
10	160
11	80
Heat treatment	
Control	640
80°C/60 min	640
90°C/60 min	160
100°C/60 min	160
121°C/15 min	320
Storage time	
Control	1280
-20°C/3-15 days	1280
4°C/3-15 days	1280
30°C/3-15 days	1280
37°C/3-15 days	1280
-20°C/30 days	1280
4°C/30 days	1280
30°C/30 days	640
37°C/30 days	640

3.4.6 Purification of bacteriocin

The bacteriocin produced by *Lc. lactis* KTH0-1S was purified by a four-step method: ammonium sulfate precipitation, RP-C_8 cartridge, cation-exchange chromatography and RP-HPLC. The antibacterial activity, yield, fold purification of bacteriocin KTH0-1S are summarized in Table 9. RP-HPLC analysis revealed that a distinct peak at the retention time of 19.6 min (peak B) was obtained (Fig. 19). This RP-HPLC purified fraction was individually collected and, when tested, exhibited activities against *P. pentosaceus* DMST 18752 used as indicator strain. It was used for further molecular weight determination through tricine SDS-PAGE and MS/MS analysis. The active peptide band in Tricine SDS-PAGE displayed an approximate molecular weight between 3.5 and 6.5 kDa (Fig. 20A). This result was confirmed by MS/MS analysis showing a sharp peak corresponding to 3.346 kDa (Fig. 20B), and clear zone in native PAGE developed after overlaying with indicator strain that corresponded to this antibacterial peptide. However, it has been reported that *Lc. lactis* strains produced four nisin variants, namely nisin A (3.350 kDa), nisin Z (3.333 kDa), nisin Q (3.332 kDa) and nisin F (3.315 kDa), that differed in both amino acid composition and biological activity (Piper *et al*., 2011).

Several researches found that the nisin Z differed from nisins A and F with respect to one amino acid residue (De Kwaadsteniet *et al*., 2008; Yoneyama *et al*., 2008; Piper *et al*., 2011). For example, nisin A has histidine (H) instead of asparagine (N) at position 27, whereas nisin F has valine (V) instead of isoleucine (I) at position 30 in peptide chain of nisin Z (Fig. 21). Nisin Q differs from nisin Z by having valine (V) and not alanine (A) at position 15 and valine (V) instead of isoleucine (I) at position 30 (Fig. 21) (Piper *et al*., 2011). The inhibitory activity of nisin variants exhibit similar antimicrobial spectra. Previous study reported that nisin F had highly specific activity against *S. aureus* than other nature variants of nisin (Piper *et al*., 2011). Additionally, nisins A and Z are more easily oxidized than other nisins caused by methionine residues at position 17 and 21 in their peptide chain can receive two oxygen atoms in an oxidizing environment (Yoneyama *et al*., 2008). Yoneyama *et al*. (2008) found that the degree of oxidization in nisin molecules leads to the reduction of antimicrobial activity.

Table 9 Purification steps of bacteriocin produced by *Lc. lactis* KTH0-1S.

Purification steps	Total volume (ml)	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg protein)	Recovery (%)	Fold
CFS	1000	640,000	10,670.0	59.9	100.0	1
70% AMP	100	512,000	394.2	1,298.8	80.0	21.7
C_8 cartridge	100	256,000	42.0	6,095.2	40.0	101.8
Cation Exchange	30	153,600	7.8	19,692.3	24.0	328.8
Desalting	14	143,360	7.3	19,654.5	22.4	328.1
Semi-prep HPLC	27	138,240	2.0	70,137.0	21.6	1,170.9

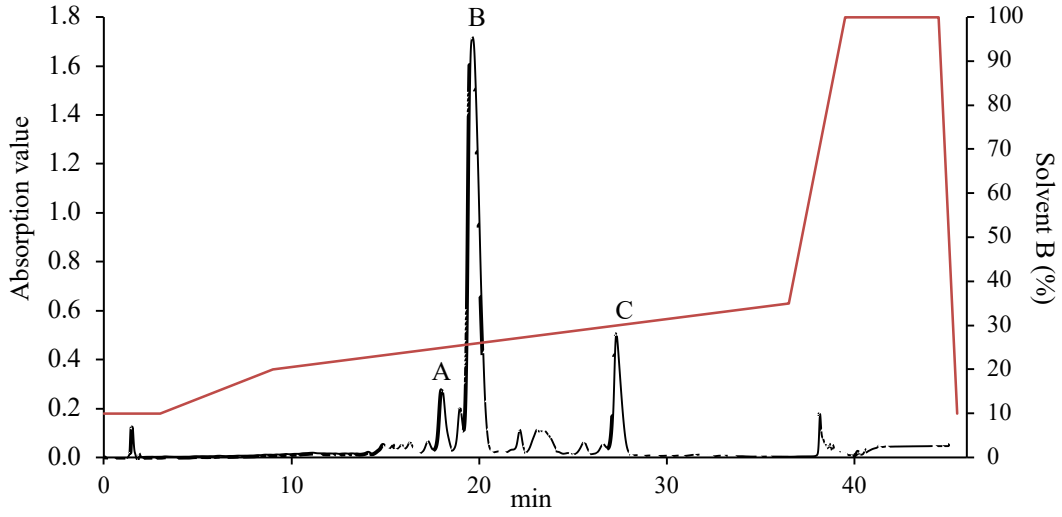

Figure 19 HPLC chromatogram of partially purified bacteriocin from *Lc. lactis* KTH0-1S. The bacteriocin was applied to RP-HPLC and eluted using a linear gradient of 20-35% of solvent B (20% IsOH/ 80% ACN/ 0.03% TFA).

PCR amplification with primers targeting known bacteriocins and sequencing could identify nisin genes involving bacteriocin production. The amplification result generated a positive band for nisin (Fig. 20C) and the sequence analysis of nisin structural gene was 100% identical to nisin Z because the substitution of asparagine residue (ATT) instead of histidine at position 27 (Fig. 22) in the peptide chain indicated bacteriocin produced by *Lc. lactis* KTH0-1S was nisin Z.

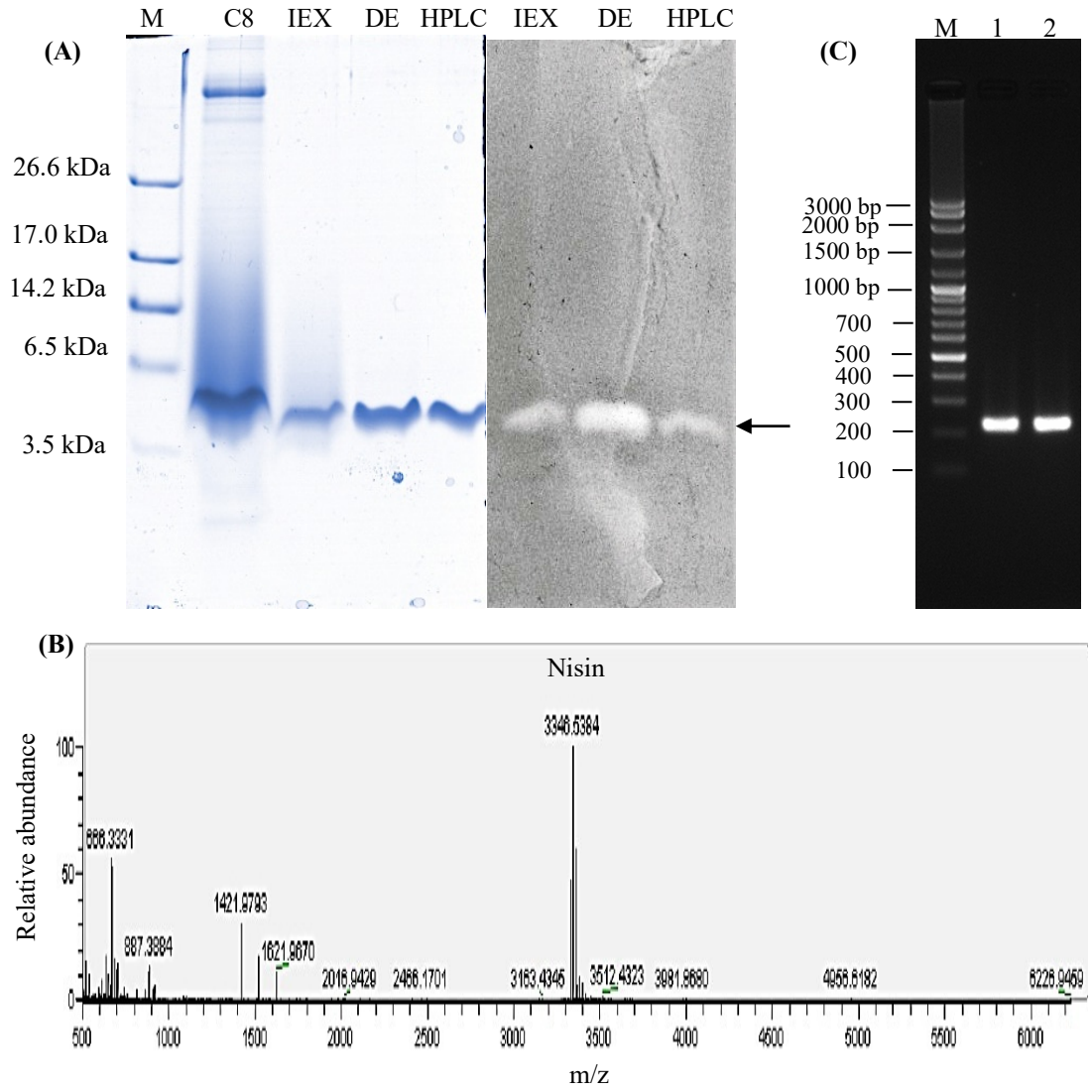

Figure 20 Tricine SDS-PAGE and inhibition zone of bacteriocin produced by *Lc. lactis* KTH0-1S. Lane M; an ultra-low molecular weight marker, Lane C8; PPB from Sep-Pak C_8 Cartridge, Lane IEX; PPB from cation-exchange, lane DE; desalting and concentration of PPB after cation-exchange step, Lane HPLC; purified bacteriocin from RP-HPLC. (B): Mass spectrometry analysis of purified bacteriocin from *Lc. lactis* KTH0-1S. (C): The PCR products with amplified nisin gene-specific primer. Genomic DNA used as template were from: lane 1: *Lc. lactis* KTH0-1S and lane 2: *Lc. lactis* JCM 7638 (positive control). Arrow indicates peptide band corresponding antagonistic activity against indicator strain on native PAGE.

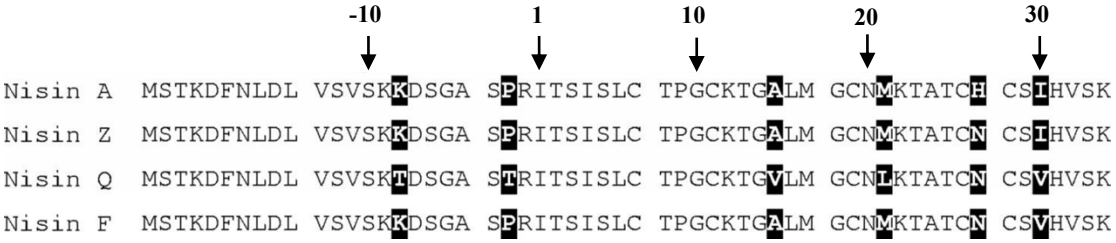

Figure 21 Amino acid sequence of nisin Z, deduced from the DNA sequence and compared to the amino acid sequences of nisin A, nisin F and nisin Q. The leader peptide of each nisin variant consist of 13 amino acids (from position -13 to -1), followed by amino acids encoding the mature protein (amino acid position 1 to 34). Differences in amino acids are indicated by letters with a black background.

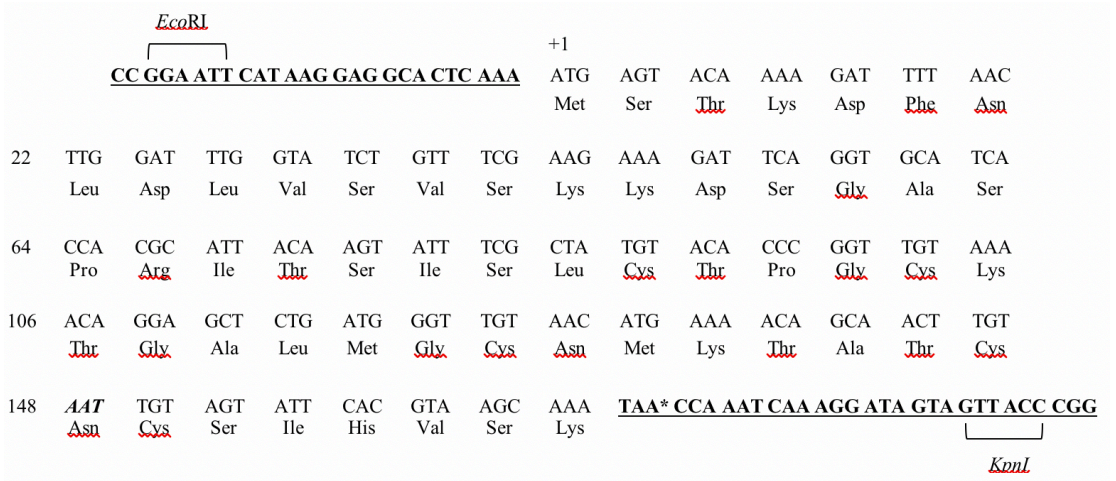

Figure 22 Nucleotide sequence and deduced amino acid sequence of the *nisZ* gene isolated from *Lc. lactis* KTH0-1S. The amino acid sequence is shown below the coding sequence. The nucleotide of *nisZ* sequence in bold and italic was asparagine residue (ATT) at position 27. Primers are in bold and underline. Stop codon is shown by asterisk.

Nisin Z is a lantibiotic containing thioether-bridged amino acids: lanthionines and methyl-lanthionines, their amino acids are responsible for nisin Z properties such as acid tolerance, heat stable and board spectrum inhibitory activity (Chatterjee *et al*., 2006; Sequeiros *et al*., 2015). These findings revealed that bacteriocin produced by strain KTH0-1S is identical to nisin Z (Fig. 21). The properties of antibacterial substance are interesting because nisin Z is the most important and single commercially available antimicrobial peptide that has already been approved by Food

and Drug Administration (FDA) as bio-preservative for human food (FDA, 1988). Therefore, nisin Z-producing *Lc. lactis* KTH0-1S could be possibly applied as bio-preservative agent in food products.

3.4.7 Safety evaluation

The application of *Lc. lactis* KTH0-1S as natural barrier or competitive exclusion bacterium requires preliminary evidences to support its safety. Although, *Lc. lactis* has the advantage of being GRAS and was given QPS status by the European Scientific Committee (EFSA, 2007; EFSA, 2011b). This strain should be characterized to be absolutely certain of the absence of the any risk factors for safe in human consumption. The absence of hemolytic activity, antibiotic resistance and any risk factors are considered as a safety prerequisite for the selection of LAB strains to be used in foods (FAO and WHO, 2002). According to the results from hemolysis testing, native strain *Lc. lactis* KTH0-1S exhibited γ-hemolytic activity (no hemolysis) (Fig. 23A) comparing with positive control (*S. aureus* DMST 8840) (Fig. 23B). This is in agreement with *Lc. lactis* isolated from Sardinian dairy products showing non-hemolytic activity on sheep blood agar (Cosentino *et al*., 2012).

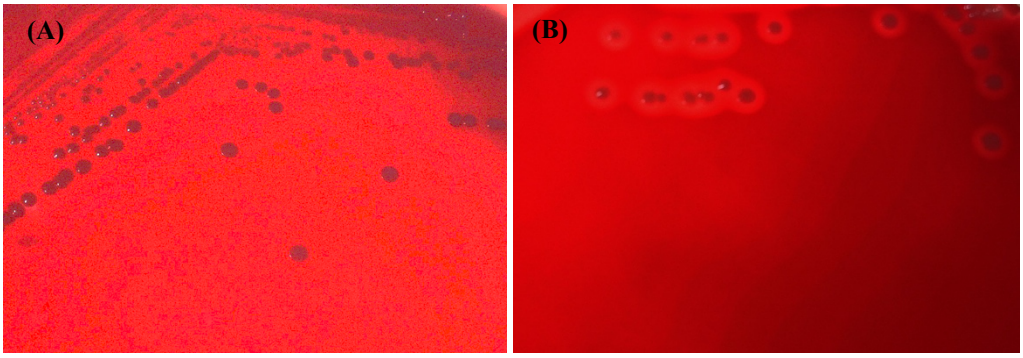

Figure 23 Appearance of hemolytic activity of *Lc. lactis* KTH0-1S (γ-hemolytic) (A) and *S. aureus* DMST 8840 (B) (β-hemolytic on blood agar).

The antibiotic susceptibility of *Lc. lactis* KTH0-1S was considered sensitive when MICs breakpoints suggested by EFSA (2012) are used. The different profiles of antibiotics susceptibility of lactococci have been documented in many publication (Hummel *et al*., 2007; Fernández *et al*., 2011; Cosentino *et al*., 2012; Hwanhlem *et al*., 2013). *Lc. lactis* KTH0-1S was sensitive to other clinically important antibiotics such as ampicillin, clindamycin, chloramphenicol, erythromycin,

tetracycline and vancomycin (Table 10). This obtained result is in accordance with the studies of Rodríguez-Alonso *et al*. (2009) and Ishihara *et al*. (2013). Several studies reported the susceptibility of *Lc. lactis* to erythromycin, chloramphenicol and vancomycin, but revealed resistance to clindamycin, tetracycline and streptomycin (Flórez *et al*., 2007; Zycka-Krzesinska *et al*., 2015). A study by Poelarends *et al*. (2002) presented that *Lc. lactis* could express the multidrug resistant transports protein (LmrA and LmrP) that both confer a resistance to many clinically antibacterial agents (macrolides, streptogramins and tetracycline). Thus, different *Lc. lactis* strains may containing drug resistance genes which could transfer them to other bacteria (Toomey *et al*., 2010). Consequently, these data indicate that strain KTH0-1S has no transferable drug-resistant genes to other bacteria present in food products.

Table 10 Antibiotic susceptibility of *Lc. lactis* KTH0-1S.

Antibiotics	**MIC (µg/ml)**	**MIC breakpoint[a] (µg/ml)**	**Antibiotic susceptibility[b]**
Ampicillin	1	2	S
Chloramphenicol	4	8	S
Clindamycin	0.25	1	S
Ciprofloxacin	8	4	n.r.
Erythromycin	>0.5	1	S
Kanamycin	32	64	S
Streptomycin	32	32	S
Tetracycline	1	4	S
Vancomycin	0.5	4	S

MIC, minimum inhibitory concentration.
n.r. is not required.
[a] The MIC breakpoints were chosen as suggested by EFSA (2012).
[b] R: resistant and S: sensitive.

A virulence factor is an effector molecule that increases the ability of microorganisms to cause disease beyond that intrinsic to the species background (Eaton and Gasson, 2001; Omar *et al*., 2004; Semedo *et al*., 2003; Vankerckhoven *et al*., 2004). Genotypical tests for virulence factors using PCR technique indicated that *Lc. lactis* KTH0-1S was negative for carrying virulence genes (*ace, asa1*, *cylA, cylB, efaAfs, esp* and *gelE*) when compared with the positive controls as shown in Fig 24. Checking of these virulence genes in this strain was of foremost importance because *Enterococcus* and *Lactococcus* are closely related, and enterococci are often the causative agents of infections in hospitalized patients (Vankerckhoven *et al*., 2008). As previous studies reported that high frequencies of positive results were observed for *asa1*, *ace* and *esp*

in *Lactococcus* spp. isolates (Biscola *et al*., 2014; Perin *et al*., 2014). These genes are related to the production of different substances enrolled in the microbial colonization and adhesion at biotic and non-biotic surfaces, and also related to evasion of the host immune system (Eaton and Gasson, 2001; Vankerckhoven *et al*., 2008).

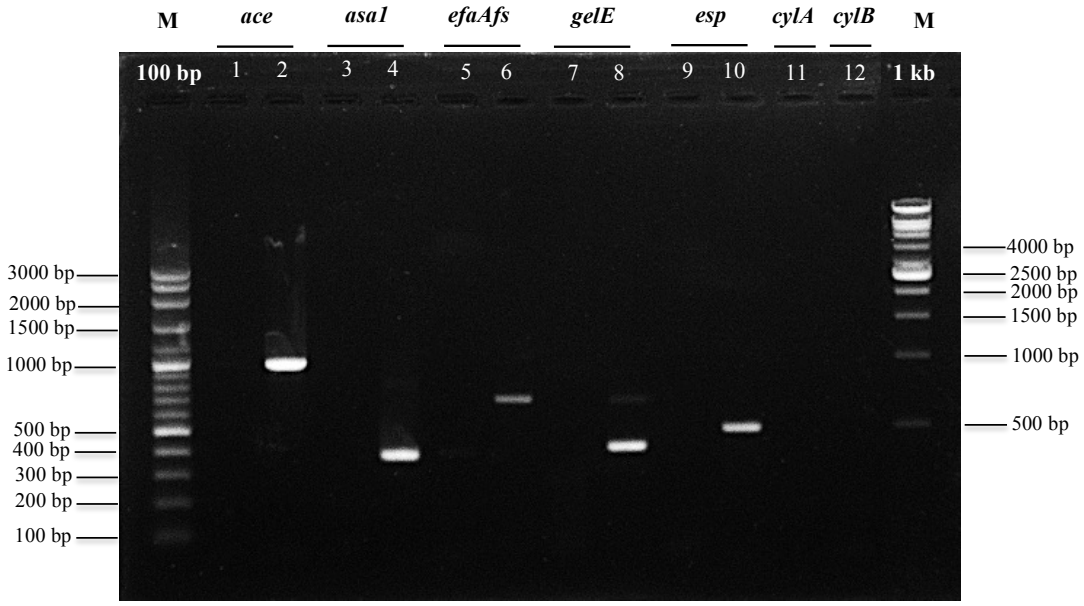

Figure 24 The presence of virulence genes in *Lc. lactis* KTH0-1S was detected by PCR. Lanes 1, 3, 5, 7, 9 11 and 12: amplification products of corresponding genes obtained for *Lc. lactis* KTH0-1S; lanes 2, 4, 6, 8 and 10 amplification products of corresponding genes obtained for positive controls.

Furthermore, the occurrence of biogenic amines (cardavarine, histamine, putrescine and tyramine) in fermented foods are concern to consumers and health authorities due to their toxicological effects. Biogenic amines have been classified regarded as potentially hazardous compounds of food that may cause disorders to consumers (EFSA, 2011a). As the results of TLC and PCR detection of biogenic amines production clearly demonstrated that *Lc. lactis* KTH0-1S was non-biogenic amines production (cardavarine, histamine, putrescine and tyramine) (Fig. 25A), and did not carry on the decarboxylase genes (*ldc*, *hdc*, *odc* and *tdc*) as compared with the biogenic amines producer strains (Fig. 25B). Several studies reported that high frequencies of tyramine production were positive in *Lactococcus* spp. isolated from fermented foods (Buňková *et al*., 2009; Perin *et al*., 2014; Flasarová *et al*., 2016). Tyramine is considered as a most toxic and particularly relevant for food safety that

found in many fermented foods such as cheese, fermented sausages and fermented fish (Buňková *et al*., 2009; Tabanelli *et al*., 2014). Six hundred milligram of tyramine is a toxic level to expose an adverse effect in human, whereas 50 mg of tyramine can be acute headache and hypertensive crisis in patients under treatment with taking mono-amino oxidase inhibitor (MAOI) drugs (EFSA, 2011a). Accordingly, these results demonstrate that nisin Z-producing *Lc. lactis* KTH0-1S is safe as a possible alternative candidate LAB for application as starter culture or protective culture to eliminate the high-risk microorganisms from fermented foods.

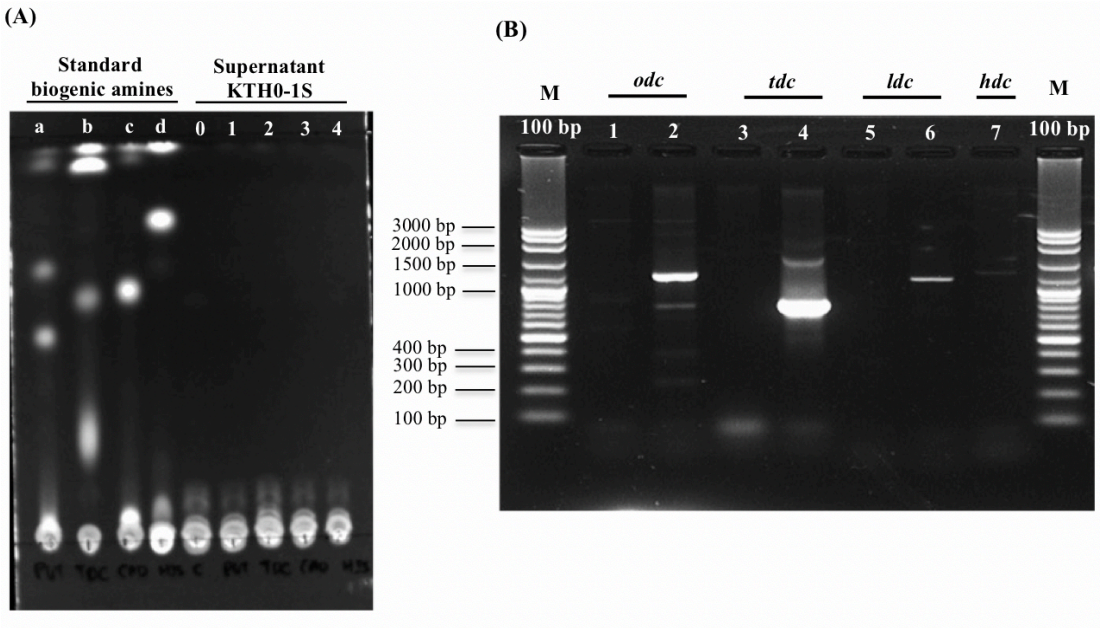

Figure 25 (A): The dansylated biogenic amines standards were separated by TLC: putrescine (a), tyramine (b), cadavarine (c) and histamine (d). CFS of bacteria grown in M17 medium containing: (0) without substrate, (1) lysine, (2) tyrosine, (3) ornithine and (4) histidine were dansylated and separated on TLC plate. (B): PCR amplification of amino acid decarboxylase genes in *Lc. lactis* KTH0-1S. Lanes 1, 3, 5, and 7: PCR products of corresponding biogenic amines genes obtained for *Lc. lactis* KTH0-1S; lane 2: Amplification of *odc* gene from the putrescine-producing strain (*Ent. faecium* CE5-1; 1440 bp); lane 4: The 825-bp fragment of *tdc* gene within a tyramine-producing strain (*Lb. pentosus* K2N7); lane 6: The 1185-bp fragment of *ldc* gene within a cadaverine-producing strain (*S. aureus* DMST 8840).

3.5 Conclusion

The bacteriocin-producing LAB isolated from traditional products can be promising strains to be employed as bio-control agents to prevent food-poisoning outbreak caused by *S. aureus*. Our finding has shown that nisin Z-producing *Lc. lactis* KTH0-1S exhibited inhibitory effect against food-borne pathogens and food spoilage bacteria. In addition, nisin Z KTH0-1S has heat-stable and wide rang of pH stability. The growth of *S. aureus* decreased considerably when cultivated with this strain and its bacteriocin. Furthermore, this strain was sensitive to all antibiotics tested and did not harbor virulence genes. Therefore, isolated and studied *Lc. lactis* KTH0-1S is a good candidate for application as starter or protective culture reducing the risk of staphylococcal food-poisoning.

CHAPTER 4

REDUCTION OF TYRAMINE ACCUMULATION IN *KUNG-SOM* BY NISIN Z PRODUCING *LACTOCOCCUS LACTIS* KTH0-1S AS STARTER CULTURE

4.1 Abstract

Tyramine production of 20 strains of lactic acid bacteria and coagulase-negative staphylococci isolated from Thai traditional fermented shrimp (*kung-som*) was investigated by using chromatographic and molecular methods. Among 20 strains tested, *Enterococcus faecalis* 9Y, *Enterococcus faecalis* D0KS13 and *Weissella cibaria* D0KS11 were able to produce tyramine and also possess the tyrosine decarboxylase (*tdc*) gene. This indicated a positive correlation existing between the presence of *tdc* gene and production of tyramine. The effectiveness of nisin Z-producing *Lactococcus lactis* KTH0-1S against tyraminogenic strains was evaluated in co-cultivation. Both co-culture of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 with *Lc. lactis* KTH0-1S decreased significantly the growth rate and the maximal cell concentration, depending on the initial inoculum level of tyraminogenic strain. The reduction of tyramine accumulation decreased strongly when tyraminogenic strain was inoculated at 4.0 log CFU/ml and, to a lesser extent, at 5.0 and 6.0 log CFU/ml. In addition, *kung-som* inoculation with nisin Z-producing *Lc. lactis* KTH0-1S as a starter culture could suppress the growth of undesirable bacteria such as Enterobacteriaceae, *Staphylococcus aureus* and total viable count, comparing with spontaneous fermented *kung-som*. Tyramine was predominant amine formed during *kung-som* fermentation. The addition of *Lc. lactis* KTH0-1S as a starter culture in *kung-som* was reducing accumulation of tyramine about 100 mg/kg in comparison with naturally fermented *kung-som*. Hence the use of bacteiocinogenic amine-negative strain as a starter culture is advisable for production of safer *kung-som* with decreasing pathogen contaminants and lowering the tyramine content.

4.2 Introduction

Biogenic amines (BAs) are organic substances, and formed mainly by amino acid decarboxylation, which widely spread in food spoilage and food-related microorganism (Torriani *et al*., 2008; Toy *et al*., 2015). The most relevant of BAs is that consumption of food containing the high concentration levels which could cause harmful to human health. Indeed, the concentration of BAs in food has been used as the food quality marker. The relevant BAs in food and fermented food are histamine, tyramine, putrescine and cadaverine, which occur by microbial decarboxylation of precursor amino acids such as histidine, tyrosine, ornithine and lysine, respectively. Among them, histamine and tyramine are determined as the most toxic and tyramine is the most abundant BAs in fermented foods, particularly in protein-rich fermented products including cheeses (Leroy and De Vuyst, 2010), fermented sausages (Tosukhowong *et al*., 2011) and fishery products (Zhang *et al*., 2013). European Food Safety Authority (EFSA, 2011a) reported that the high level of 600 mg/kg tyramine in foods exerts toxic effects on human health such as trigger migraines, gastric and intestinal problems and allergic responses in sensitive people. The consumption of 6 ppm tyramine could produce a slight reaction, whereas its presence at 50 ppm can cause severe headaches and hypertensive crisis in patients under treatment taking mono-amino oxidase inhibitor (MAOI) drugs (EFSA, 2011a).

The accumulation of BAs in foods is strongly related to the quality of raw materials, the amount of free amino acids, effect of starter culture, the presence of aminobiogenic bacteria and processing condition (Komprda *et al*., 2004; Gardini *et al*., 2016). The reduction of tyramine or decrease of other BAs accumulation in foods, and especially in fermented foods is attracting the increased attention of researchers and international organization. In fermented food, the use of bacteriocin producing lactic acid bacteria (LAB) as non BAs-producing starter cultures is an effective strategy reducing the formation of most dangerous BAs (tyramine and histamine) (Gao *et al*., 2014b; Wang *et al*., 2015). The reduction of BAs can be based on the inhibition of the growth of aminobiogenic microorganisms or inhibition of their decarboxylase activities. The possible application of bacteriocin producing LAB as protective cultures has been tested in dairy product (Leroy and De Vuyst, 2010), in fermented sausage (Lu *et al*., 2015), in fermented fish and in other food products (Khan and Oh, 2016). In

particular, a nisin producer *Lactococcus lactis* is able to limit tyramine and histamine production by *Enterococcus faecalis* and *Streptococcus thermophilus* (Tabanelli *et al*., 2014). Many publications reported that the inoculation of bacteriocinogenic LAB starter cultures have been tested successfully in fermented products to prevent excessive tyramine accumulation (Tosukhowong *et al*., 2011; Zhang *et al*., 2013; Kongkiattikajorn, 2015; Sun *et al*., 2016).

Kung-som is a traditional Thai fermented shrimp that is popularly consumed in the south of Thailand because of its special taste (Hwanhlem *et al*., 2010). During fermentation-microbial growth, acidification, and proteolysis provide suitable conditions for tyramine production (Gardini *et al*., 2016). The fermented shrimps or shrimp by products have been reported to be rich in essential amino acids, especially the tyrosine (10.78 mg/g dry mass) (Sánchez-Machado *et al*., 2008) as a precursor of the tyramine, which can reach concentrations exceeding 1,000 mg/kg constituting an obvious health hazard. In our previous study, nisin Z-producing *Lc. lactis* KTH0-1S exhibited the antagonistic activities against food-borne and food-spoilage bacteria. This strain was sensitive to clinically used antibiotics and non BAs production (Saelao *et al*., 2017). This strain was selected as a potential starter culture improving the safety of *kung-som*. Accordingly, the objectives of the present study were to screen the tyramine-producing bacteria isolated from *kung-som* and to evaluate the effectiveness of the inhibition of tyramine-producing bacteria and reduction of tyramine accumulation *in vitro* by nisin Z-producing *Lc. lactis* KTH0-1S. Additionally, the influence of non BAs-producing *Lc. lactis* KTH0-1S as a starter culture on tyramine accumulation, microbiological properties and pH during the fermentation of *kung-som* was studied.

4.3 Materials and Methods

4.3.1 Bacterial strains and culture conditions

Bacteriocinogenic *Lc. lactis* KTH0-1S stocked in glycerol (-20 °C) was subcultured twice in M17 broth (Merck, Germany) at 30°C for 24 h used as an inoculum. Ten LAB strains originated from *kung-som* were cultivated in de Man-Rogosa-Sharpe (MRS; Hi-media, India) (bacilli) broth or M17 broth (Merck, Germany) (cocci) broth at 30°C for 24 h. Ten strains of coagulase negative staphylococci (CNS) isolated from *kung-som* was cultivated in tryptone soya broth (TSB; Hi-media, India) broth and then incubated at 30°C for 24 h. For routine analysis, the strains were

activated by successive transfer in the respective media at 30°C for LAB and CNS before use.

4.3.2 Screening and identification of tyramine-producing bacteria isolated from *kung-som*

The bacterial strains isolated from *kung-som* were tested for their capability to produce the tyramine initially with the plate method described by Bover-Cid and Holzapfel (1999). Only LAB and CNS strains, which gave positive reaction on plate assay were selected to confirm tyramine formation using thin layer chromatography (TLC) analysis as proposed by Kim and Kim (2014). LAB strains showing positive result (purple color) were activated in MRS (bacilli) or M17 (cocci) broth overnight, while CNS strains were cultivated in TSB at 30°C for 24 h. The active culture of each LAB isolate was inoculated into MRS or M17 broth supplemented with 0.5% (w/v) L-tyrosine disodium salt (Sigma-Aldrich, Steinheim, Germany). Amines were converted into their fluorescent dansyl derivative according to the method described by Landeta *et al*., (2007a). The derived samples were spotted on TLC plate (silica gel 60 F_{254}, Merck, Darmstadt, Germany). As positive control 2% (w/v) tyramine stock standard solution in 40% ethanol (Sigma-Aldrich, Steinheim, Germany) was prepared. The dansylated samples were separated on TLC plate by chloroform/di-ethyl ester/tri-ethylamine (3/2/1, v/v/v) and were sprayed with isopropanol/tri-ethanolamine (4/1, v/v) (Saelao *et al*., 2017). The fluorescent dansyl derivative on TLC plate was visualized under UV light. The LAB or CNS strains showing positive results were selected to further determine the tyramine concentration using high performance liquid chromatography (HPLC) analysis as described below.

The presence of gene encoding the tyrosine decarboxylase enzymes (*tdc*) involved in tyramine formation was detected by PCR with specific primers (TDC-f: 5′-TGGYTNGTNCCNCARACNAARCAYTA-3′) and (TDC-r: 5′-ACRTAR TCNACCATRTTRAARTCNGG-3′) (de las Rivas *et al*., 2006). The PCR amplification was performed in 25 µl volume containing 2.5 µl of 2 µM of each primer, 12.5 µl of DreamTaq Green PCR Master Mix (Thermo-scientific, MA, USA), 2 µl of DNA template and volume was adjusted with DNase free water. PCR program was applied to an initial denaturing at 95°C for 15 min, followed by 36 cycles at 95°C for 1 min, at 53°C for 1 min, and at 72°C for 3 min with final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel containing

1×SYBR Safe (Invitrogen™, CA, USA) in 0.5×Tris-Acetate-EDTA (TAE; pH 8) buffer and observed using UV trans-illuminator with gel documentation (UVItec, England).

4.3.3 Competition between bacteriocinogenic *Lc. lactis* KTH0-1S and tyramine-producing strains

The effectiveness of nisin Z-producing *Lc. lactis* KTH0-1S against tyramine-producing strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11) was studied in co-cultivation as described by Tabanelli *et al*. (2014) with some modifications. The competition between *Lc. lactis* KTH0-1S strain and each tyramine-producing strain was studied in MRS broth supplemented with 0.5% (w/v) tyrosine as precursor. Competition study was evaluated by inoculating *Lc. lactis* KTH0-1S (at a level of about 7.0 log CFU/ml) in the presence of different initial inoculum levels of the tyraminogenic strains (approx. 4.0, 5.0 and 6.0 log CFU/ml). Non-competition (control) study was obtained by inoculating separately of tested medium with either *Ent. faecalis* D0KS13 or *W. cibaria* D0KS11 at different initial inoculum sizes (4.0, 5.0 and 6.0 log CFU/ml) and cultivated at 30°C for 48 h. The growth of *Lc. lactis* KTH0-1S, *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 were periodically monitored at regular intervals for 48 h by plating the viable cells on selective condition for different species. Specifically, *Lc. lactis* KTH0-1S was enumerated onto M17 incubated at 20°C for 72 h, whereas *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 were plated onto mannitol salt agar (Hi-Media, India) (Quiloan *et al*., 2012) and MRS agar respectively, incubated at 45°C for 24 h. Additionally, the bacteriocin activity was determined by agar well assay (Saelao *et al*., 2017) by using both of the indicator strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11). The measurement of the tyramine content was performed by HPLC analysis as described below.

4.3.4 Application of bacteriocinogenic *Lc. lactis* KTH0-1S used as a starter culture for *kung-som* production

4.3.4.1 Preparation of starter culture and *kung-som* fermentation

For the preparation of starter culture, 100 µl of glycerol stock of nisin Z-producing *Lc. lactis* KTH0-1S was inoculated in 10 ml of M17 at 30°C and incubated overnight. One-milliliter of the active culture was transferred to 30 ml of M17 broth and incubated at 30°C for 24 h. Cells were harvested by centrifugation (10,300 × g) for 15 min at 4°C and washed twice with 30 ml of sterile saline (0.85%). The cell pellets

were resuspended in 30 ml of sterile saline and then, used as a starter culture, inoculated in *kung-som*.

Small shrimp were bought in Songkhla Province, Thailand. Samples were kept on ice and transported in ice pack to the Prince of Songkla University. Upon arrival, samples were immediately washed three times with water and divided into two equal portions. The original formula of *kung-som* consisted of 1 kg shrimp, 75 g NaCl, 300 g sugar and 250 ml water. The mixtures were transferred to glass jar and covered with polyethylene film. Rubber band was used to hold the film in place. Two different *kung-som* were prepared with starter culture (at final concentration of starter culture 10^7 CFU/g) and without starter culture. Both were incubated at 30°C for 10 days. Periodically, samples were taken for analysis of chemical and microbiological properties as well as tyramine content by HPLC.

4.3.4.2 Chemical and microbial determination

Aliquots were taken from mother solution for the measurements of the pH, total acidity, and lactic acid and tyramine content (HPLC analysis as described below) at days 1, 2, 3, 4, 5, 6, 8 and 10. At each sampling time, a 50-g portion of *kung-som* samples was aseptically weighed into 450 ml of sterile peptone water (0.1% peptone, Hi-media, India) in a sterile bag and blended with a stomacher (Seward, England) for 2 min. The serially ten-fold dilution was prepared, and then 0.1 ml of appropriate dilution plated on the specific media to quantify different bacteria groups as described by Wang *et al*. (2015) with some modification. Total viable counts (TVC) were determined by plate count agar after incubation at 30°C for 48 h. The lactococci and lactobacilli counts were plated on M17 and MRS agar after incubation at 30°C for 48 h under anaerobic condition, respectively. Enterobacteriaceae counts were enumerated by plating the viable cells on violet red bile agar (VRBA, Hi-media, India) at 37°C for 48 h. *S. aureus* counts were cultured on Baird Parker agar (Hi-media, India) supplemented with egg yolk tellurite emulsion at 37°C for 48 h.

4.3.4.3 Amines extraction and dansyl chloride (DnsCl) derivatization

The BAs extraction was performed according to Kongkiattikajorn (2015) with minor modifications. Five grams of *kung-som* samples were added in 10 ml of 10% (w/v) trichloroacetic acid (TCA, Merck, Germany) and then shaken at 150 rpm for 1 h. The samples were further centrifuged at 10,300×*g* for 15 minutes. After centrifugation, the supernatant was collected for dansyl-derivative formation.

The BAs derivatization was performed as described by Smělá *et al*. (2003) with slight modification. Four hundred microliters of the supernatant (or standard) was mixed with 0.2 ml of saturated sodium bicarbonate (Na_2CO_3) solution. Four hundred microliters of dansyl chloride reagent (DnsCl, Merck, Germany; 5 mg/ml in acetone) was added to the mixture and then shaken for 1 min. Derivatization preformed for 1 h in dark at 55°C. After derivatization, 100 µl of 25% ammonia hydroxide (NH_4OH) solution was added to eliminate the excessive DnsCl. Hydrophobic amine derivatives were extracted by 0.5 ml of diethyl ether for 2 times. Finally, the organic phase was evaporated to dryness under nitrogen, the solid residue was dissolved in 0.5 ml of acetonitrile (ACN). The solution was filtered through a nylon membrane filter 0.2 µm and stored at -20°C until the HPLC analysis.

4.3.4.4 Tyramine determination using HPLC analysis

Tyramine in *kung-som* samples was determined by HPLC (HP 1200; Agilent Technologies, Waldbronn, Germany) after DnsCl derivatization. Twenty microliters of sample were separated by RP-HPLC with Symmetry® RP-C8 column (100 mm × 4.6 mm, particle size 3.5 µm; Waters, Milford, MA, USA). The gradient elution (Table 11 for elution profile) was done with solvent A (MilliQ water, 0.03% trifluoroacetic acid (TFA) and solvent B (ACN, 0.03% TFA). Twenty microliters of sample were injected and monitored at a wavelength of 254 nm. The amount of tyramine was expressed as mg/ml by reference to a calibration curve obtained with tyramine standard.

Table 11 HPLC elution program.

Time (min)	DnsCl		Flow rate (ml/min)
	Solvent A*	Solvent B	
0	55	45	1.0
2	55	45	1.0
10	30	70	1.0
12	20	80	1.0
13	0	100	1.2
15	0	100	1.2
16	55	45	1.2
18	55	45	1.0

*Solvent A = MiliQ water + 0.03% trifluoroacetic acid (TFA), Solvent B = acetronitrile (ACN)+ 0.03% TFA.

4.3.4.5 Growth modeling

Microbial growth for each treatment and strain were performed separately by fitting data to the Baranyi model (Baranyi & Roberts, 1994) using DMFit program in the ComBase browser (http://www.combase.cc).

$$y(t) = y_0 + \mu_{\max} A(t) - \ln\left(1 + \frac{e^{\mu_{\max} A(t) - 1}}{e^{(y_{\max} - y_0)}}\right)$$

where

$$A(t) = t + \frac{1}{\mu_{max}} ln\,(e^{\mu_{max} t} + e^{-h_0} - e^{(-\mu_{max} t - h_0)})$$

Where: $y(t)$ is the log of cell concentration at time t [h] (CFU/ml); y_0 is log of the initial cell concentration (CFU/ml); y_{max} is log of the maximum cell concentration; μ_{max} is the maximum specific growth rate in the exponential phase (log CFU/ml/h), and h_0 is a measure of the initial physiological state of cells and expresses the work necessary for the cells to adapt to the environmental condition. In particular, $h_0 = \mu_{max}\lambda$, where λ is lag time (h).

4.3.4.6 Statistical analysis

All data in this study represented the mean of three independent replicates. Statistical analyses of result were performed with analysis of variance (ANOVA) followed by Duncan's test using Statistical Package for the Social Science (SPSS 16.0 for windows, SPSS Inc, IL, USA). A value of $p<0.05$ was considered to be statistically significant. Moreover, model quality was evaluated by the coefficient of determination (R^2) and their performance was checked by the root mean square error.

4.4 Results and Discussion

4.4.1 Screening and identification of tyramine-producing bacteria isolated from *kung-som*

Kung-som is a traditional Thai fermented shrimp obtained by fermentation of endogenous microorganisms. LABs were the major microflora, while CNS were minor bacterial flora in *kung-som* products (Saelao *et al*., 2016). Their activity can be dominated by lactic acid fermentation and by degradative phenomena. Even though they were present in low amounts they could generate undesirable BAs. Maijala and Eerola (1993) concluded that the contaminated food-spoilage bacteria and LAB play an important role in histamine and tyramine accumulation during

fermentation. In this case, all LAB in *kung-som* products should be considered to be natural strains present as contaminant in raw ingredients and their development leads to tyramine production during *kung-som* fermentation. Accordingly, in the present study the tyramine-producing strains were isolated from *kung-som* because knowledge of the potential tyramine producers is essential to avoid their inclusion or to inhibit their unfavorable propagation during *kung-som* production.

Twenty bacterial strains originating from *kung-som* were screened first for their abilities of tyramine production on selective medium plate (Fig. 26) and TLC analysis. Fourteen of bacterial strains (6 LAB and 8 CNS strains) exhibited positive reactions on decarboxylase plates (purple color present or halo zone around the colonies) were selected to confirm the tyramine formation by TLC assay. Some studies have described the false-positive reactions in agar media testing due to the formation of different alkaline compounds such as ammonia and not tyramine. These compounds can be a reason for the detection of false-positive colonies on selective agar plate (Bhardwaj *et al*., 2009).

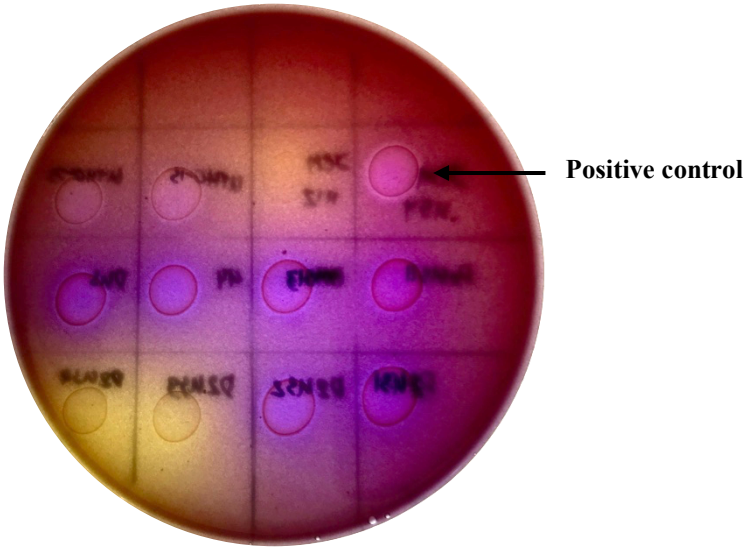

Figure 26 Screening plate method for tyramine production by using Bover-Cid and Holzapfel medium. Arrow indicates tyramine-producing strain (*Lb. sakei* JCM 1157).

TLC method has a higher sensitivity for tyramine detection (*i.e.*, 10 mg/l) than the plate assay and is simple and inexpensive (Garcia-Moruno *et al*., 2005). The TLC showed that culture broth from three isolates (9Y, D0KS13 and D0KS11) yielded positive band on TLC plate (Fig. 27) similar with tyramine-producing positive

strain (*Lb. sakei* JCM 1157), while other LAB and CNS strains gave negative results. The tyramine producer strains (9Y, D0KS13 and D0KS11) were able to produce 226.89±6.58, 241.69±6.74 and 234.96±3.95 mg/l of tyramine in the medium after 3 days of cultivation as detected using HPLC analysis, respectively.

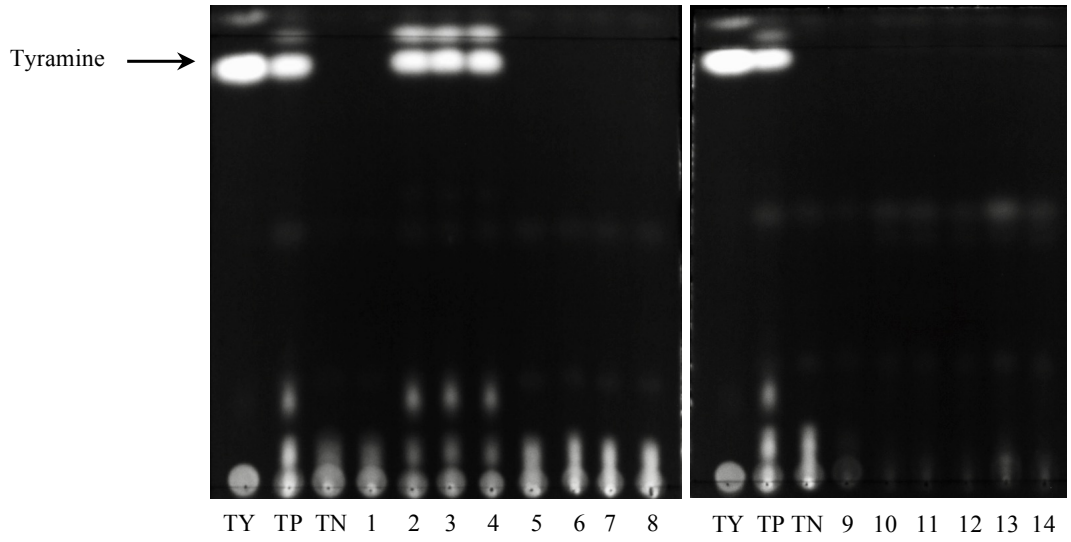

Figure 27 The tyramine production was verified by TLC method after positive result on decarboxylation agar of the tested strains with 0.5% tyrosine added as a substrate. Lane TY: tyramine standard solution; lane TP: *Lb. sakei* JCM 1157 (positive control); lane TN: *Lc. lactis* KTH0-1S (negative control); lane 1: *Lc. lactis* KTH0-2S; lane 2: *Ent. faecalis* 9Y; lane 3: *Ent. faecalis* D0KS13; lane 4: *W. cibaria* D0KS11, lane 5: *Lb. plantarum* D2KS11; lane 6: *Lb. plantarum* D2KS12; lane 7: *S. carnosus* S21K22; lane 8: *S. condimenti* K8S5, lane 9: *S. condimenti* K16S2, lane 10: *S. condimenti* K21S9, lane 11: *S. piscifermentans* K2S12; lane 12: *S. piscifermentans* K5S15, lane 13: *S. piscifermentans* K8S21, lane 14: *S. xylosus* K2S6.

Additionally, the PCR assay was performed to confirm results obtained from TLC and HPLC whether they harbor the tyrosine decarboxylase (*tdc*) gene. It was found that all 3 tyramine-producing strains contained the positive amplicon corresponding to the expected size of the internal fragment of *tdc* gene (825 bp) (Fig. 28). By comparing the full length of 16S rDNA sequence, the isolates 9Y and D0KS13 were identified as *Ent. faecalis* (99% identity), while the 16S rDNA amplification of D0KS11 presented 99% similarity with the 16S rDNA sequences reported for *W. cibaria* in the GenBank database. These rDNA sequences (*Ent. faecalis* 9Y,

D0KS13 and *W. cibaria* D0KS11) were deposited in DDBJ/EMBL/GenBank databases with accession numbers LC279528, AB685880 and AB685884, respectively.

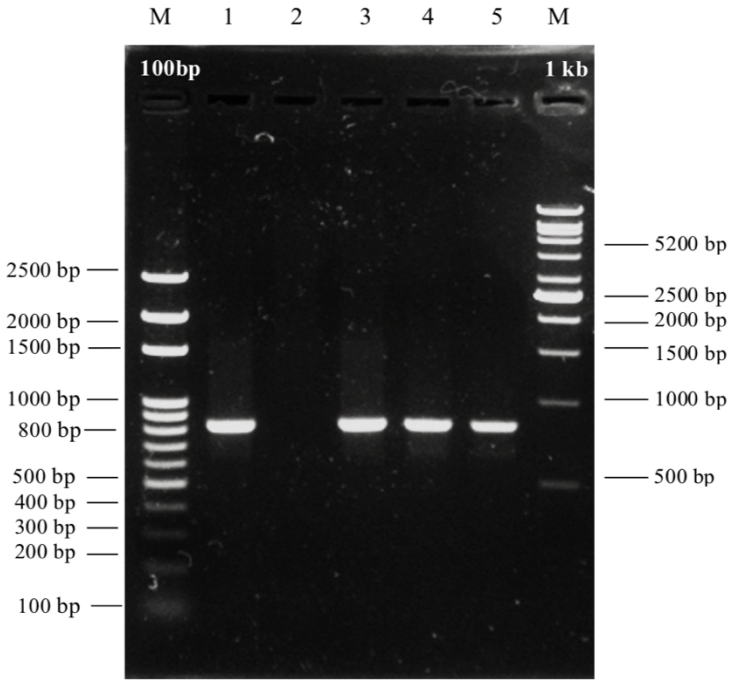

Figure 28 The presence of tyrosine decarboxylase (*tdc*) gene in tyraminogenic strain isolated from *kung-som* was amplified by PCR using specific primers (expected size 825 bp). Lane 1: *Lb. sakei* JCM 1157 (positive control); lane 2: *Lc. lactis* KTH0-1S (negative control); lane 3: *Ent. faecalis* 9Y; lane 4: *Ent. faecalis* D0KS13, lane 5: *W. cibaria* D0KS11 and lane M: 100 bp and 1 kb DNA molecular weight marker (Vivantis, Malaysia).

Tyramine production is a characteristic of certain microbes such as Enterobacteriaceae, enterococci, lactobacilli and lactococci (Bhardwaj *et al*., 2009; Kim and Kim, 2014). However, the ability of bacteria to generate the BAs depends on the species, strain and environment (Kim and Kim, 2014; Elsanhoty and Ramadan, 2016). *Enterococcus* sp. are generally considered as having an ambiguous status in respect to food safety. Indeed, although they are LAB, they have neither Qualified Presumption of Safety (QPS) nor Generally Regarded As Safe (GRAS) status (Ladero *et al*., 2012). Hence, they need to check the absence of virulence factors and tyramine production before their use in food processing. Previous studies reported that tyramine-producing enterococci were present in many kinds of food such as cheese, kimchi,

fermented meat, fermented sausage, and winery products (Tosukhowong *et al*., 2011; Ladero *et al*., 2012; Kim and Kim, 2014; Nisiotou *et al*., 2015). Several authors reported that *Ent. faecalis* strains isolated from meat and dairy products were able to produce tyramine and putrescine simultaneously. Their presence has been related to the accumulation of BAs in food products (Ladero *et al*., 2012).

Weissella sp. have been reported to be able to produce putrescine (Pereira *et al*., 2009; Nisiotou *et al*., 2015), whereas it lacked the ability of tyramine production. In our best knowledge, this is the first report revealing the presence of *W. cibaria* with tyramine-synthesizing capacity isolated from *kung-som.* Capacity confirmed by chromatographic and PCR methods. Our results indicated that isolated strains of *Ent. faecalis* 9Y, *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 exhibited the ability of tyramine production and they were selected for further study. Nisin Z-producing *Lc. lactis* KTH0-1S against tyramine-producers with respect to tyramine production was studied using a co-culture method as described below.

4.4.2 Competition between bacteriocinogenic *Lc. lactis* KTH0-1S and the tyramine producing strains

Using the nisin Z-producing LAB as potential protective culture to prolong shelf-life and ensure the safety as well as quality of foods has been reported by several authors (Leroy and De Vuyst 2010; Siroli *et al*. 2016). The nisin Z-producing *Lc. lactis* KTH0-1S was found to be safe with non BAs-producing ability in previous work (Saelao *et al*., 2017) and it was confirmed as a tyraminogenic inhibitor against *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 *in vitro* in the present study. These obtained data were modeled using the equation of Baranyi and Roberts (1994) and its parameters are shown in Table 12.

In addition, the growth curve and tyramine production of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 at different initial cell concentration with or without nisin Z-producing *Lc. lactis* KTH0-1S as a competitor are shown in Fig. 29 (A-F). In the presence of the nisin Z-producing *Lc. lactis* KTH0-1S in all treatments, both the growth rate (μ_{max}) and final cell concentration (y_{max}) of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 decreased significantly when compared with non-competitive treatments (Table 12).

Table 12 Estimates of the model parameters of Baranyi and Roberts (1994) describing the population dynamics of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11, inoculated at different initial concentrations, grown alone or in competition with nisin Z-producing *Lc. lactis* KTH0-1S (at a concentration of 7 log CFU/ml). The parameters are reported with the standard error (within brackets), residual mean square error (RSME) and correlation coefficient (R).

Parameter	Tyramine producing strains initial inoculum at 4 log CFU/ml			
	Ent. faecalis DKS13 without competition	*Ent. faecalis* DKS13 *Lc. lactis* KTH0-1S	*W. cibaria* DKS11 without competition	*W. cibaria* DKS11 *Lc. lactis* KTH0-1S
y_0	3.93 (±0.16)	4.03 (±0.05)	3.93 (±0.12)	4.04 (±0.09)
μ_{max}	0.25 (±0.02)	0.15 (±0.01)	0.26 (±0.02)	0.18 (±0.01)
h_0	0.03 (±0.02)	0.18 (±0.01)	0.12 (±0.04)	0.32 (±0.02)
y_{max}	9.82 (±0.11)	5.99 (±0.02)	9.85 (±0.16)	6.57 (±0.06)
RMSE	0.177	0.045	0.423	0.086
R^2	0.994	0.996	0.990	0.991
Parameter	Tyramine producing strains initial inoculum at 5 log CFU/ml			
	Ent. faecalis DKS13 without competition	*Ent. faecalis DKS13 Lc. lactis KTH0-1S*	*W. cibaria DKS11 without competition*	*W. cibaria DKS11 Lc. lactis KTH0-1S*
y_0	5.08 (±0.10)	5.07 (±0.06)	5.08 (±0.10)	5.07 (±0.06)
μ_{max}	0.23 (±0.01)	0.19 (±0.02)	0.23 (±0.01)	0.19 (±0.02)
h_0	0.03 (±0.01)	0.34 (±0.04)	0.03 (±0.01)	0.39 (±0.01)
y_{max}	9.93 (±0.08)	7.64 (±0.04)	9.90 (±0.08)	7.77 (±0.04)
RMSE	0.120	0.073	0.117	0.067
R^2	0.996	0.995	0.996	0.996
Parameter	Tyramine producing strains initial inoculum at 6 log CFU/ml			
	Ent. faecalis DKS13 without competition	*Ent. faecalis DKS13 Lc. lactis KTH0-1S*	*W. cibaria DKS11 without competition*	*W. cibaria DKS11 Lc. lactis KTH0-1S*
y_0	6.05 (±0.06)	6.13 (±0.11)	6.08 (±0.06)	6.05 (±0.12)
μ_{max}	0.25 (±0.01)	0.17 (±0.04)	0.26 (±0.01)	0.15 (±0.03)
h_0	0.16 (±0.01)	0.55 (±0.08)	0.25 (±0.01)	0.25 (±0.06)
y_{max}	10.12 (±0.04)	7.9 (±0.08)	10.09 (±0.04)	7.94 (±0.08)
RMSE	0.082	0.127	0.078	0.116
R^2	0.998	0.966	0.998	0.969

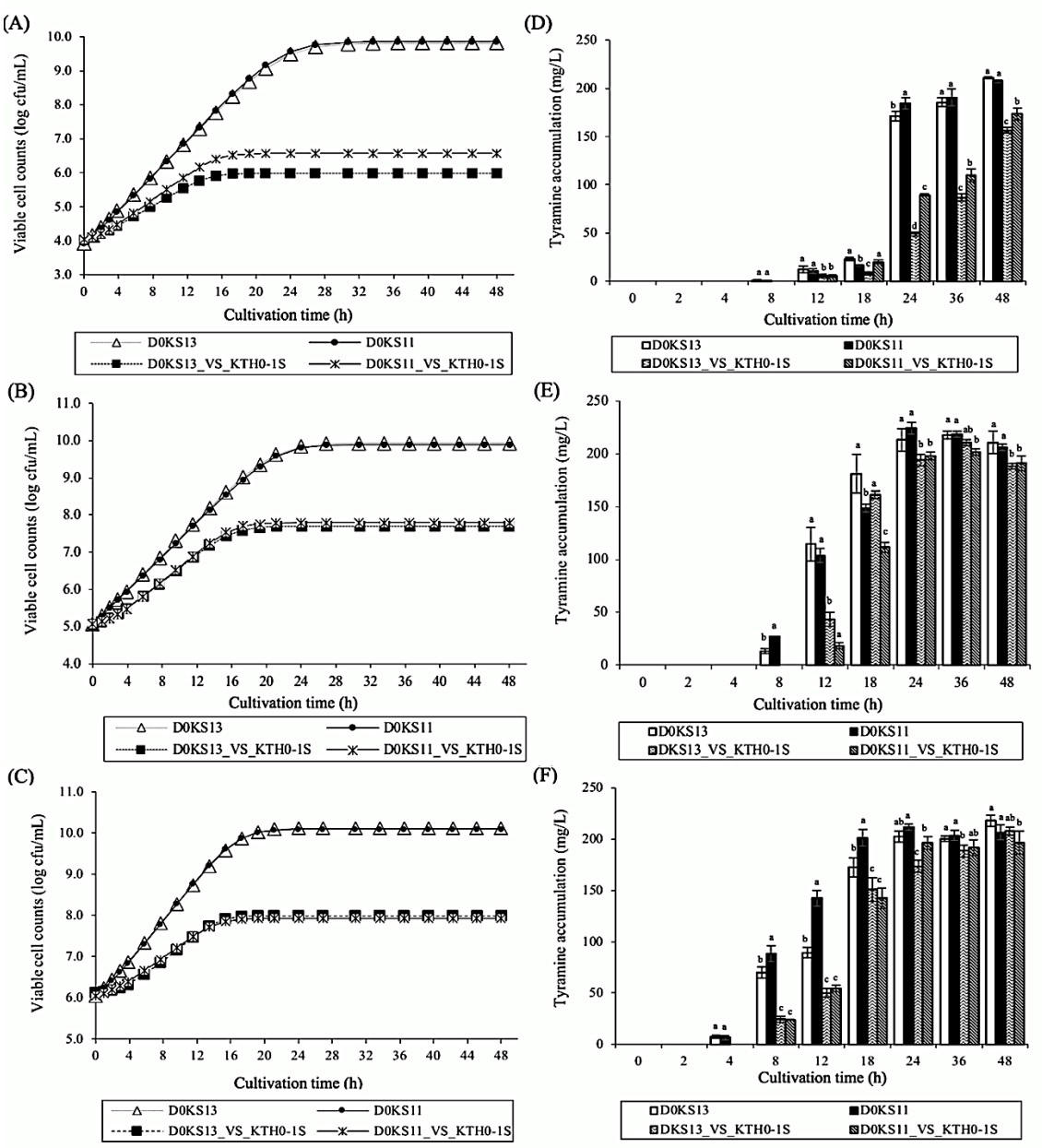

Figure 29 The growth curves (according to the parameters from Table 12) and tyramine production of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 with or without of competitive culture by nisin Z *Lc. lactis* KTH0-1S. The graph (27A-C) are referred to initial concentration of 4 (A), 5 (B) and 6 (C) log CFU/ml of tyramine-producing strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11). In the figure (27D-E) the tyramine accumulated in different initial concentration of tyraminogenic strains at 4 (D), 5 (E) and 6 (F) log CFU/ml after 2, 4, 8 16, 24, 36 and 48 h of cultivation time is also reported. Different superscripts on the bars represent significant differences (p<0.05).

The final cell concentration of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11 in co-culture were reduced by 2.1-3.8 log CFU/ml and 2.1-3.2 log CFU/ml, respectively in comparison with their controls (Fig 29A-C). Also, the final population decrease of the inoculated tyramine-producing strain was also observed during co-culture exhibiting the highest reduction at lowest inoculum (4.0 log CFU/ml) of tyramine-producing strain. This inhibitory action in competitive culture was result of the inhibitory activity of bacteriocin produced by *Lc. lactis* KTH0-1S exhibiting an increase of the antagonistic activity during cultivation period, which reached a maximum (40 AU/ml) at 24 h incubation time (Fig. 30). Additionally, the inhibitory effect was due to the effect of competition for nutrients. More rapidly growing nisin Z-producing *Lc. lactis* KTH0-1S may generate lack of a limiting nutrient for tyraminogenic bacteria what remains in agreement with the result of Tabanelli *et al*. (2014) who reported that the presence of bacteriocin-producing lactococci in starter culture (majority population) was decreasing the cell density of tyramine producing *Ent. faecalis* EF37.

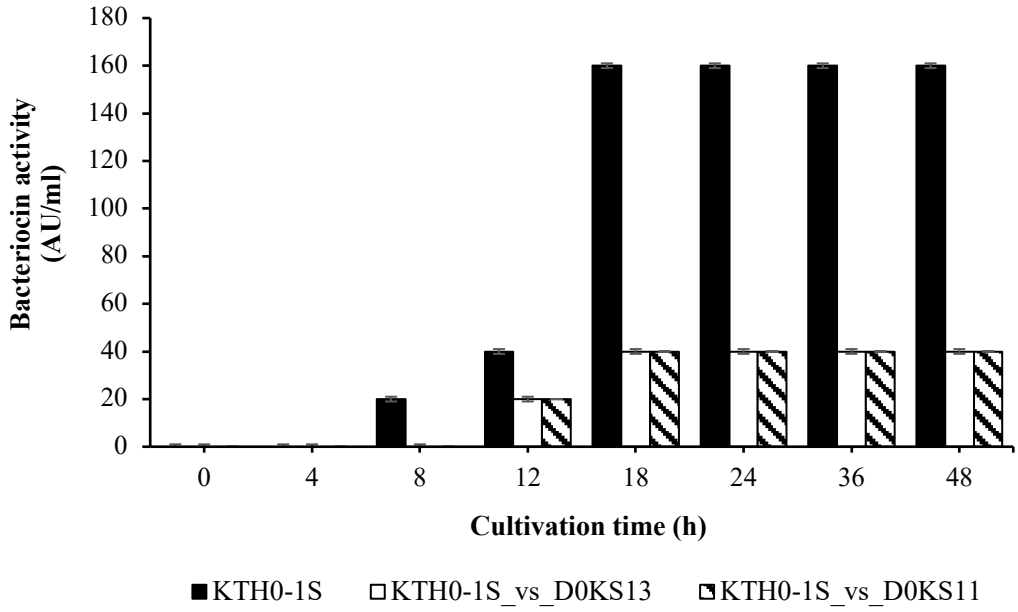

Figure 30 Bacteriocin activity was expressed in competitive and non-competitive (control) treatments. Bar represent the standard deviation from three determination.

The accumulation of tyramine at different initial cell concentrations alone or in the presence nisin Z *Lc. lactis* KTH0-1S shown in Fig. 29D-F. The tyramine content was independently the initial tyramine-producing strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11) inoculum, tyramine content reached a final concentration of about 200 mg/l after 48 h of cultivation time. The tyramine concentration did not significantly change when the cultivation was extended up to 72 h. Tyramine accumulation during competitive treatments was significantly (p<0.05) smaller than those of control treatments (Fig. 29D-F). At the lowest initial cell concentration of *Ent. faecalis* D0KS13 and *W. cibaria* D0KS11, the final amount of tyramine when inoculated with *Lc. lactis* KTH0-1S was reduced by 30-50 mg/l in comparison with the control sample, while tyramine decreased with minor efficacy at higher initial inoculum of tyramine-producing strains at the end of cultivation. The reduction of tyramine in co-culture might be due to the tyrosine as substrate was depleted to be a limiting factor for tyramine formation (Kongkiattikajorn, 2015).

Accordingly, the pH level (5.0-5.3) in competition culture (Fig. 31A-C) was lower than the control (5.8-5.9) at the end of incubation time. This could be also the cause of the reduction of tyramine amount in co-cultures, in agreement with result of Gardini et al. (2001) who reported that BA production significantly decreased when pH decreasing lower than 6.0. Several researchers reported that an acidity condition prohibited the bacterial amino acid decarboxylase activity involving BA formation (Gardini *et al*., 2001; 2016). Despite this inhibition, the tyraminogenic cells remained viable in the medium and could accumulate tyramine in co-cultivation.

Our results suggest that inhibitory effect in competition treatments depended largely, if not totally, on bacteriocin (nisin Z) secreted by *Lc. lactis* KTH0-1S. It is also possible that there was a combined synergy of low pH, secondary metabolites and restricted substrate amount. Additionally, the tyramine reduction might be due to the decrease of tyrosine decarboxylase activity or less favorable for enzyme activity intracellular conditions (Torriani *et al*., 2008), resulting in different mechanisms of action by nisin Z-producing *Lc. lactis* KTH0-1S.

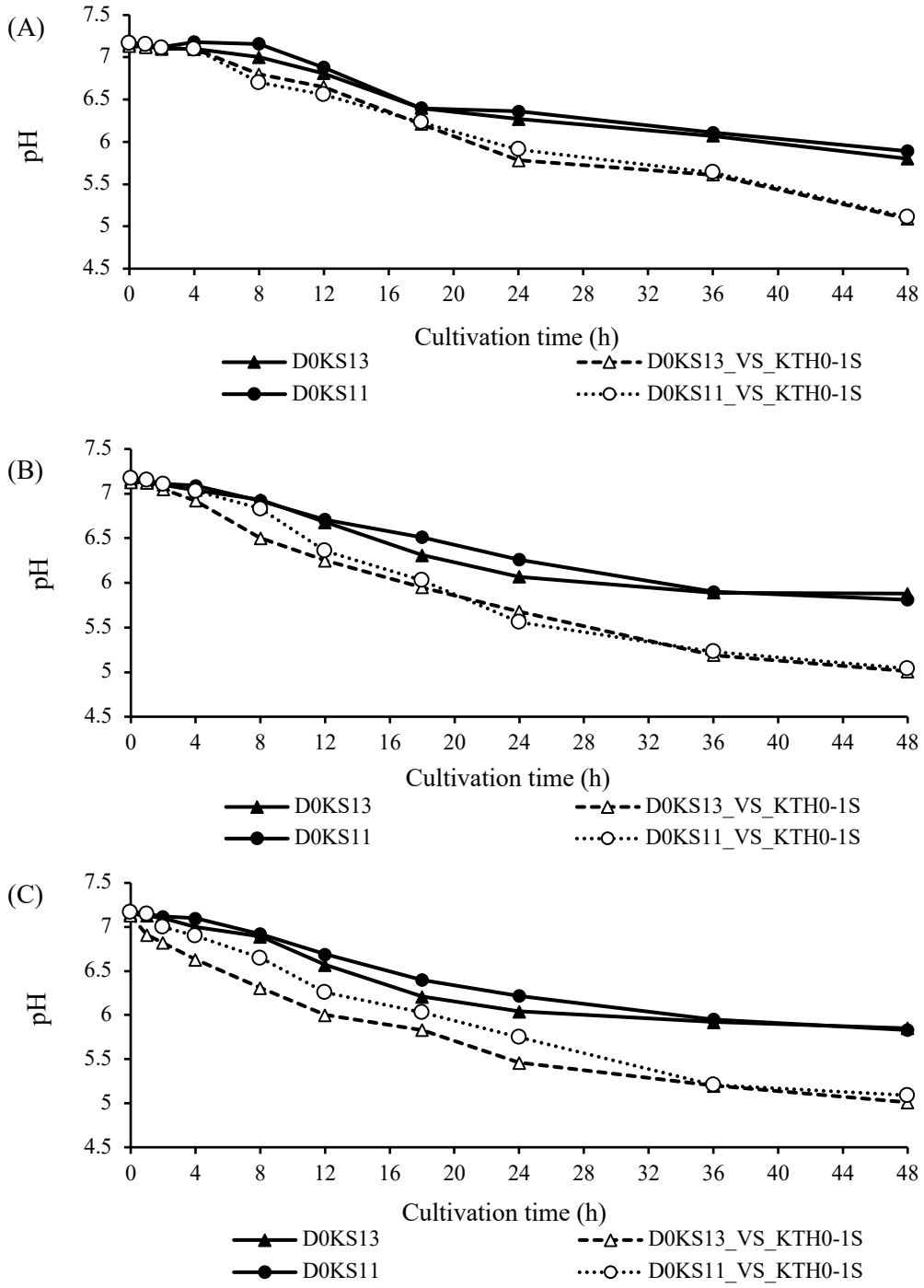

Figure 31 pH value of the competition between *Lc. lactis* KTH0-1S strain and each tyramine-producing strain was studied in MRS broth supplemented with 0.5% (w/v) tyrosine as precursor. The initial concentration of 4 (A), 5 (B) and 6 (C) log CFU/ml of tyramine-producing strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11).

4.4.3 Control of tyramine accumulation by nisin Z-producing *Lc. lactis* KTH0-1S as starter culture in *kung-som* fermentation

For the safety increase in *kung-som* production, *Lc. lactis* KTH0-1S was applied as a starter reducing the growth of undesirable bacteria, hence diminishing the BAs accumulation in this product. pH in *kung-som* decreased after the second day of fermentation. pH of the *kung-som* inoculated with *Lc. lactis* KTH0-1S (batch S) decreased faster than in *kung-som* spontaneously fermented (batch C). Nevertheless, pH and lactic acid production of both finished *kung-som* did not differ a lot (Fig. 32). The amounts of lactococci, lactobacilli, Enterobacteriaceae, *S. aureus* and total viable count (TVC) were monitored during fermentation of *kung-som* from with and without starter culture treatments and are shown in Table 13. LAB (cocci) counts in *kung-som* with a starter culture (batch S) increased rapidly, and reached level up to 9.4 log CFU/g, which was significantly higher than spontaneously fermented *kung-som* (batch C) (Table 13). Whereas, the lactobacilli in batch C showed significantly lower (p<0.05) than batch S inoculated with a starter at early stage of fermentation. In this line, the use of bacteriocinogenic *Lc. lactis* KTH0-1S as starter culture could affect the growth of lactobacilli in comparison with spontaneous *kung-som*. These differences could be the consequence of several factors such as an interaction between inter-and intra-species variabilities and adaptation of microorganism to complex matrix and effective acidification of substrate (Mangia *et al*., 2008). This result is in agreement with those reported by Aro *et al*. (2010) and Domínguez *et al*. (2016).

With regard to Enterobacteriaceae and *S. aureus* counts decreased significantly during fermentation (p<0.05), independent of the presence of starter cultures (Komprda *et al*., 2004). Enterobacteriaceae and *S. aureus* exhibited the drastic reduction occurred becoming undetectable in starter mediated *kung-som* (batch S), whereas the counts in control (batch C) was 2.5±0.04 log CFU/g at 6 days of fermentation (Table 13). Moreover, TVC counts increased during the fermentation and the inoculated *kung-som* showed significantly lower (p<0.05) TVC numbers when compared with naturally fermented *kung-som*. Our results are in agreement with previous reports in which the use of bacteriocinogenic strain as starter culture applied prior to fermentation, which could effectively inhibit the growth of Enterobacteriaceae and *S. aureus* (Lu *et al*., 2010; Gao *et al*., 2014b; Wang *et al*., 2015). The inhibitory

effect of these pathogenic bacteria might be attributed to a rapid reduction in pH and bactericidal properties of starter culture (Lu *et al*., 2010).

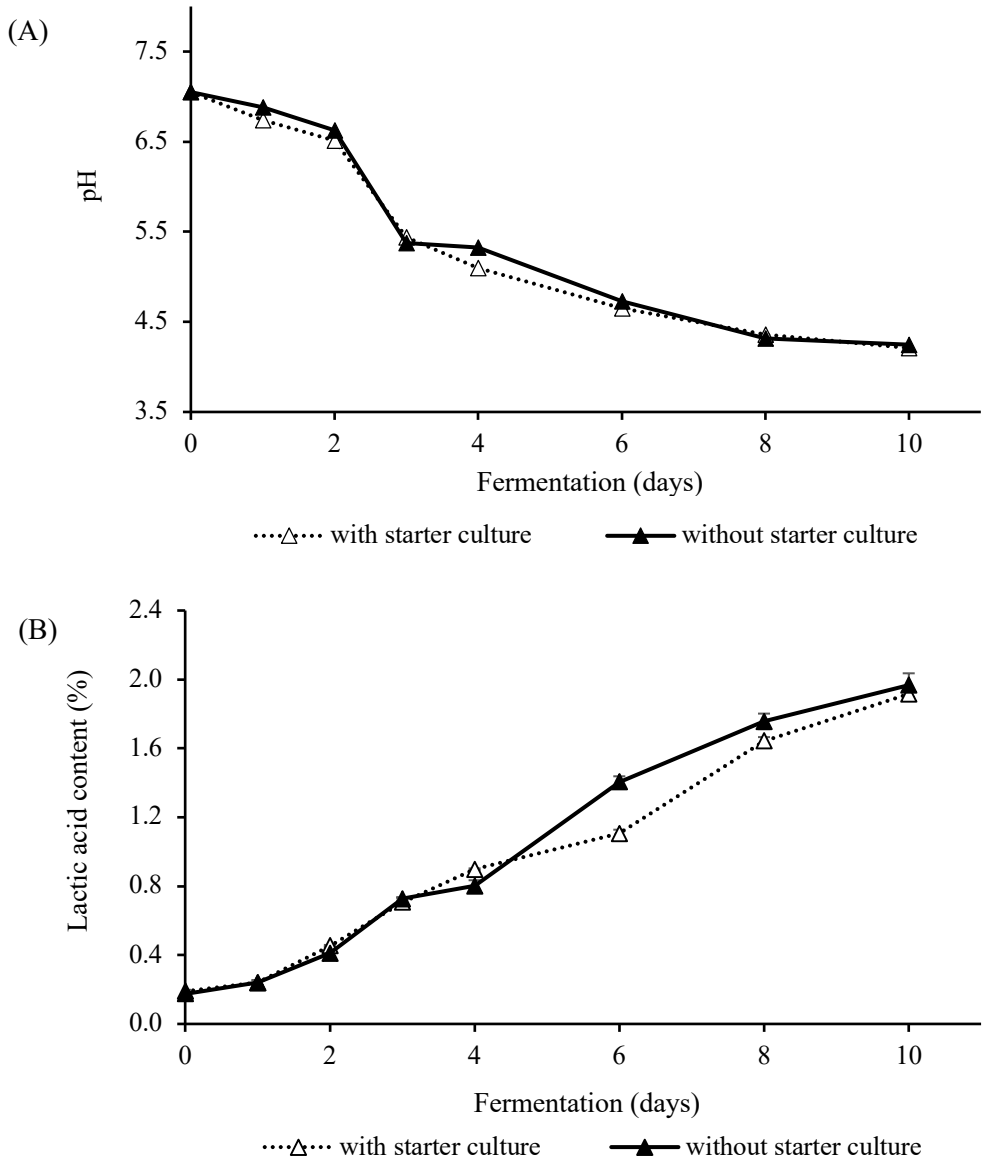

Figure 32 pH (A) and lactic acid content (%) (B) of *kung-som* produced by addition of *Lc. lactis* KTH0-1S as a starter culture and control treatment (without starter culture).

Table 13 Microbial counts (log CFU/g) during fermentation of *kung-som*.

Microbial counts	Batch[A]	Fermentation (days)							
		0	1	2	3	4	6	8	10
TVC	C	6.5±0.04[a]	8.1±0.05[b]	9.0±0.07[b]	10.1±0.03[b]	10.0±0.05[b]	8.8±0.07[a]	8.9±0.03[a]	7.8±0.12[b]
	S	6.5±0.06[a]	6.9±0.12[b]	8.3±0.04[a]	9.3±0.04[a]	9.4±0.10[a]	8.8±0.10[a]	8.9±0.06[a]	7.5±0.03[a]
LAB (bacilli)	C	5.9±0.11[a]	7.6±0.10[b]	7.7±0.05[a]	7.7±0.07[b]	8.5±0.06[b]	8.2±0.04[a]	7.6±0.04[a]	7.0±0.55[a]
	S	6.0±0.09[a]	6.6±0.10[a]	7.1±0.11[a]	7.5±0.06[a]	8.2±0.03[a]	8.7±0.04[b]	8.0±0.03[b]	7.8±0.07[b]
LAB (cocci)	C	5.0±0.07[a]	6.5±0.08[a]	8.0±0.20[a]	8.1±0.11[a]	8.3±0.07[a]	7.6±0.03[a]	7.5±0.03[a]	6.5±0.03[a]
	S	6.9±0.03[b]	7.1±0.03[b]	8.2±0.06[b]	8.9±0.04[b]	9.4±0.06[b]	8.7±0.04[b]	7.8±0.10[b]	6.6±0.06[b]
Enterobacteriaceae	C	5.5±0.03[a]	6.7±0.04[b]	6.6±0.10[b]	6.0±0.02[b]	5.8±0.07[b]	2.5±0.04[b]	nd[a]	nd[a]
	S	5.6±0.06[a]	6.6±0.06[a]	6.2±0.13[a]	5.4±0.13[a]	4.9±0.07[a]	nd[a]	nd[a]	nd[a]
S. aureus	C	3.1±0.24[a]	3.1±0.16[a]	3.5±0.17[b]	3.1±0.16[a]	3.0±0.12[b]	1.4±0.05[b]	nd[a]	nd[a]
	S	3.1±0.12[a]	2.9±0.14[a]	3.3±0.15[a]	3.1±0.13[a]	1.4±0.08[a]	nd[a]	nd[a]	nd[a]

Data are expressed mean ± standard deviation (n=3)

[a-b] Different superscripts within a column represent significant differences ($p < 0.05$); nd: non-detectable.

[A] Batch C: natural fermented *kung-som*, Batch S: *kung-som* inoculated with nisin Z-producing *Lc*. *lactis* KTH0-1S as a starter culture.

The gradual BAs formation occurred from the beginning of the fermentation, corresponded with microbial changes. Tyramine was main amine produced in *kung-som* (Fig. 33). The occurrence of tyramine in spontaneous *kung-som* was detectable after 1 days of fermentation and increased during fermentation (Fig. 34). The amounts of tyramine in *kung-som* inoculated with *Lc. lactis* KTH0-1S as a starter culture (batch S) at 10 days of fermentation were lower (p<0.05) (221.42±10.48 mg/kg) than those produced by the natural fermentation micro-flora in the control batch (C) (320.36±11.36 mg/kg), suggesting that the inoculation of nisin Z-producing *Lc. lactis* KTH0-1S as a starter culture in batch S was able to significantly (p<0.05) reduce tyramine accumulation (31%) in comparison with *kung-som* without starter culture (C). This indicates that the decarboxylase-negative starter culture can effectively inhibit the tyramine accumulation. Specifically, *Lc. lactis* KTH0-1S could be outcompete or replaced the potential aminogenic endogenous bacteria responsible for tyramine formation, thus leading to a decrease in the tyramine accumulation. This finding is in agreement with those reported by previous workers (Tosukhowong *et al*., 2011; Zhang *et al*., 2013; Latorre-Moratalla *et al*., 2014; Lu *et al*., 2015; Sun *et al*., 2016), who found that a significant effect of starter culture on tyramine contents.

Although, the amount of tyramine in spontaneous *kung-som* was exceeding 300 mg/kg, this amount was below the toxic level of tyramine established by EFSA (2011a). Low-tyramine foods cause the serious jump in blood pressure for everyone taking MAOI drugs. Therefore, our results suggest that the inoculation with nisin Z-producing *Lc. lactis* KTH0-1S as starter culture is an effective way to inhibit the growth of pathogenic (*S. aureus*) and spoilage bacteria (Enterobacteriaceae and TVC), and significantly reduce the accumulation of tyramine for avoiding the excessive dose, with results suggesting that application of bacteriocinogenic strain can be a protective culture for enhancing safety of *kung-som* and other fermented products.

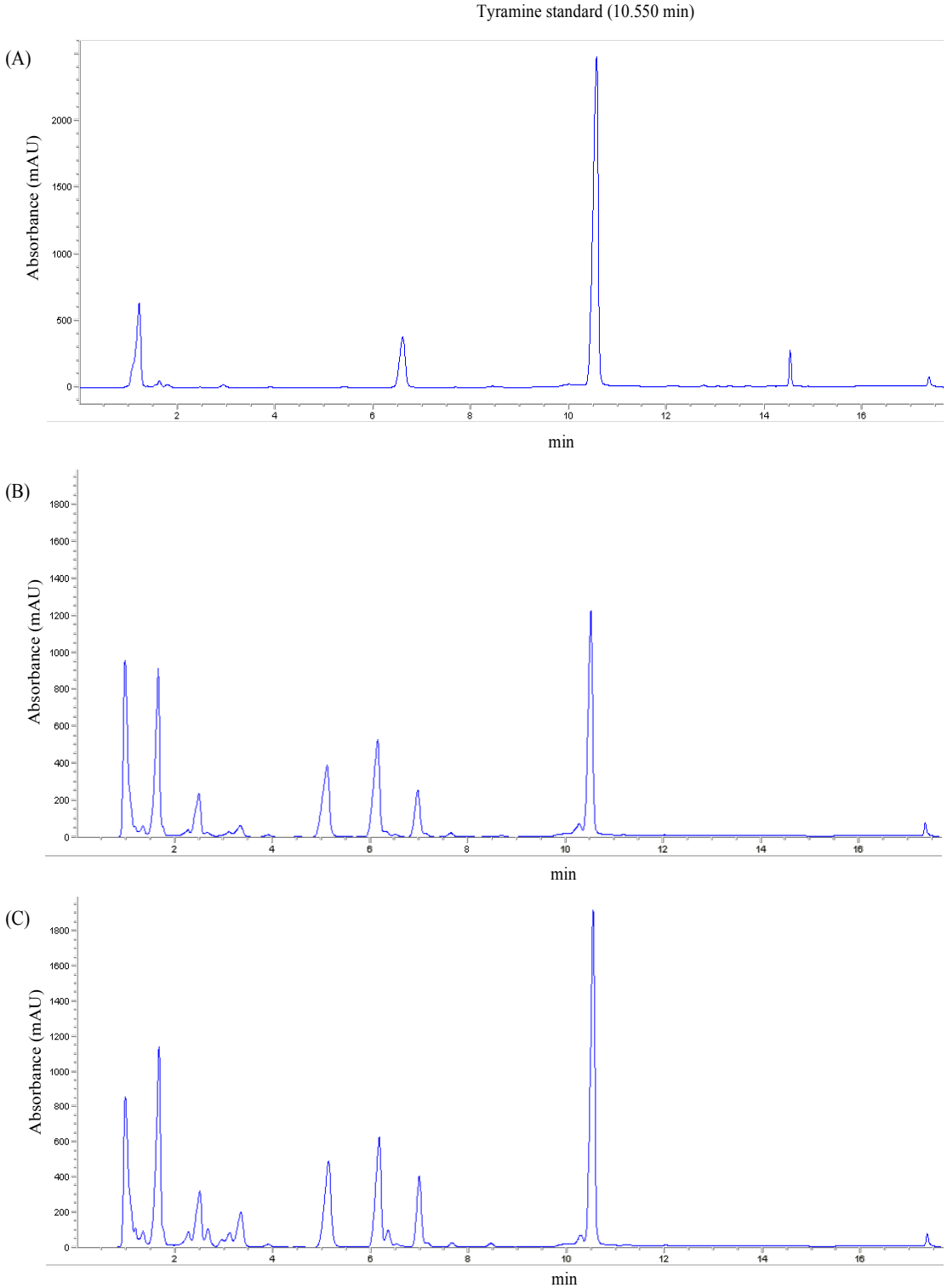

Figure 33 HPLC chromatogram of standard solution of tyramine at 250 mg/l (A); tyramine accumulation in the inoculated *kung-som* (B) and spontaneous *kung-som* (C) at 10 days of fermentation.

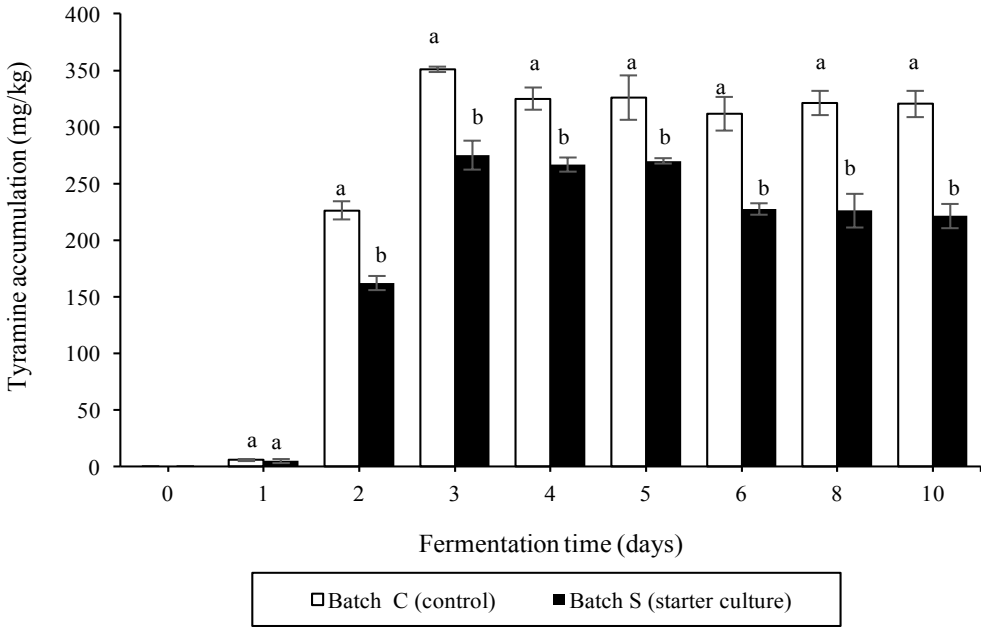

Figure 34 Amount of tyramine accumulation detected by HPLC analysis during *kung-som* fermentation, spontaneous *kung-som* (□) and inoculated of *Lc. lactis* KTH0-1S (■). Bars indicate the standard deviation from triple determinations. Different superscripts on the bars represent significant differences.

4.5 Conclusion

The occurrence of BAs is a serious problem for the safety features in many fermented products. *In situ* and simultaneous production of bacteriocin by non BAs-producing LAB present in food products constitute an advantage, both for inhibition the food-borne pathogens and reduction of BAs accumulation. Our finding demonstrates that nisin Z-producing *Lc. lactis* KTH0-1S had an inhibitory effect on the tyramine-producing strains (*Ent. faecalis* D0KS13 and *W. cibaria* D0KS11) *in vitro* and also showed the optimal ratio between nisin Z-producing strain and tyraminogenic strains with respect to the possibility to prohibit tyramine accumulation. In addition, the inoculation of non BAs-producing *Lc. lactis* KTH0-1S as a starter culture could suppress the indigenous microbiota and increase the effectiveness of the tyramine reduction during *kung-som* fermentation. Therefore, the use of amine-negative starter cultures is recommended for the production of safer *kung-som* with lowing tyramine content.

CHAPTER 5

SELECTION OF COAGULASE-NEGATIVE STAPHYLOCOCCI PRESENTING SAFETY AND TECHNOLOGICAL PROPERTIES AS STARTER CULTURE FOR *KUNG-SOM* PRODUCTION

5.1 Abstract

The aim of this study was to evaluate pathogenicity determinants and functional characteristics in coagulase-negative staphylococci (CNS) for selection of potential starter to be used in the processing of Thai fermented shrimp (*kung-som*). Among 46 CNS strains, 10 strains did not harbor *icaA* gene and non-biofilm formation, which belonged to spices: *Staphylococcus carnosus, S. condimenti, S. piscifermentans* and *S. xylosus*. In related to virulence factors, only strain *S. carnosus* K21S22 was considered as safe to be used with regard to its sensitiveness to antibiotics tested, non-hemolytic, non-toxigenic and non-aminogenic production. This strain showed the proteolytic, lipolytic and nitrate reductase activities, as well as its adaptability under the different technological conditions. For *kung-som* production, the use of autochthonous starter culture (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) was significantly affecting on microbiology safety, acidity and organoleptic characteristics in samples. The addition of only *Lc. lactis* KTH0-1S and mixed starter cultures (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) was significantly positive effect on sensory properties and safety of product when compared with control and *kung-som* inoculated with only *S. carnosus* K21S22. However, the addition of only *S. carnosus* K21S22 as starter culture provided a negative effect on *kung-som* quality. Our finding suggested that the addition of only *Lc. lactis* KTH0-1S and combination of *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as eligible starter culture for *kung-som* production could be reduced the fermentation of time periods and also increased the microbiological safety.

5.2 Introduction

Kung-som is Thai traditional fermented shrimp that widely distribute consumption in the south of Thailand. This product is produced by traditional technologies without the inoculation of starter cultures and additives. The traditional process prefers the growth of naturally microflora which affected flavors, texture, nutritional properties of fermented foods (Martín *et al*., 2007). However, it is not possible to ensure that the amount and the strains of microbial presenting in the raw material will always be the same and behave in the same way (Leroy *et al*., 2006; Bonomo *et al*., 2009). The addition of starter culture in *kung-som* production would guarantee to obtain products with repeatable hygienic and organoleptic properties in a shorter fermenting time (Sanchart *et al*., 2017). However, *kung-som* inoculated with only of LAB starter culture showed the organoleptic scores lower than those of spontaneous *kung-som* (Hwanhlem *et al*., 2010; Sanchart *et al*., 2017) because a diversity of microorganisms contribute to the development of a unique characteristic in the former.

Coagulase-negative staphylococci (CNS) are used as starter culture in the processing of many fermented products such as fermented meat (Sánchez Mainar *et al*., 2017), fermented soybean (Jeong *et al*., 2016), fermented sausage (Martín *et al*., 2007) and fermented seafood (Jeong *et al*., 2014). Several publications suggested that CNS strains, rather than LAB, play an important role to develop the organoleptic properties of fermented products through their metabolic activities (Casaburi *et al*., 2005; Martín *et al*., 2007; Müller *et al*., 2016; Sánchez Mainar *et al*., 2017). The *in situ* production of flavor compounds by starter cultures during fermentation requires CNS strains which better adapted to food environment, able to grow under the food processing. Martín *et al*. (2007) reported that selection of CNS strains from indigenous microflora would be the best selection as starter cultures for the same products because they would be more competitive than CNS from other sources, to preserve the typical characteristics of these products. In particular, *Staphylococcus carnosus* and *S. xylosus* have been reported to produce low-molecular weight substances including aldehydes, amine and free fatty acid, which have an influence on aroma compounds in fermented products (Talon *et al*., 2008; Olivares *et al*., 2011; Jeong *et al*., 2016). However, some CNS strains have shown high prevalence of antibiotic resistance and gene encoding

virulence factors to lead to the occasional opportunistic infections (Resch *et al*., 2008; Zell *et al*., 2008; Marty *et al*., 2012). The deficiency of safety data as well as history of CNS use in food fermentation in Thailand hinder their introduction for use in Thai fermented products. Therefore, CNS should be evaluated the safety properties before taking into in accounts.

The safety aspects of microbial food additives are mainly assessed by the European Food Safety Authority (EFSA, 2013). One major concern suggested in the concept of Qualified Presumption of Safety (QPS) is the existence of drugs resistance in food starter, as well as the enterotoxins production or biogenic amines in the final products. The absence of these activities would seem to be an essential criterion for selection of starter cultures (Casaburi *et al*., 2005; Jeong *et al*., 2016). Therefore, in this study to evaluate the safety aspects and technological properties of CNS isolated from *kung-som* and selected a safety and functional starter candidate. Furthermore, the effect of inoculating selected CNS strain and nisin Z-producing *Lc. lactis* KTH0-1S as starter culture on the physicochemical, microbiological, sensory properties and volatile profile using headspace solid-phase microextraction (HS-SPME) of *kung-som*.

5.3 Materials and Methods

5.3.1 Bacterial strains and media

Forty-six isolates were obtained from natural Thai fermented shrimp (*kung-som*) which identified as coagulase-negative staphylococci (CNS) bacteria using criteria of Gram-positive, spherical shape, non-motile, catalase positive and coagulase negative. All CNS isolates were used in the current study for safety assessment and technological characterization. All isolates were cultivated in tryptic soy broth (TSB; Hi-media, Mumbai, India) at 30°C for 24 h and maintained as frozen stocks held at -20°C in TSB containing 30% glycerol (w/v). Before the experiment, the CNS isolates were activated twice in the respective media and condition.

5.3.2. Safety assessment

5.3.2.1 Biofilm formation

The quantitative measurement of biofilm production was determined by using microtiter plate assay as described by Leroy *et al*. (2009). The CNS isolates were

grown overnight in TSB supplemented with 0.25% glucose at 37°C. The active culture was diluted 1:20 in fresh TSB with 0.25% glucose and then 200 µl of cell suspension was inoculated into a sterile 96-well polystyrene microplate. After 24 h incubation at 37°C, the plate was gently washed three-time with sterile phosphate buffer saline (PBS), and then stained with 0.1% crystal violet (Merck, Darmstadt, Germany) for 30 min. The wells were washed with sterile PBS again and then solubility of crystal violet in 200 µl of ethanol/acetone (80/20, v/v) was added. The absorbance at 595 nm (A_{595}) was determined by using microplate reader Powerwave X (BioTek®, VT, USA). Classification of bacterial adherence was scored as follow: -, non-biofilm forming (A_{595} ≤1); +, weak (1< A_{595} ≤2); ++, moderate (2< A_{595} ≤3); +++, strong (A_{595} > 3). All experiments were conducted in triplicate and repeated three times.

In addition, the biofilm formation ability of CNS isolates was also detected using the PCR amplification of the *icaA* gene. Previous reported, the *icaA* gene is involved in a polysaccharide intercellular adhesin synthesis and biofilm formation (Arciola *et al*., 2001). Genomic DNA of CNS isolates were extracted using a TIANamp™ Bacteria DNA kit (TIANGEN®, China) according to manufacture instruction. The PCR condition and specific primers to amplify *icaA* gene were performed according to previously described by Møretrø *et al*. (2003) (Table 14). PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel containing 1×SYBR Safe (Invitrogen™, CA, USA) and then also visualized on UV trans-illumination.

5.3.2.2 Phenotypic antibiotic susceptibility test

The non-biofilm formation strains were selected for further antibiotic susceptibility testing. The microdilution assay according to guidelines for CNS set out by Clinical and Laboratory Standards Institute (CLSI, 2012) was used. Eleven antibiotics (rang noted in µg/ml) commonly used in human and animal medicine were tested: ampicillin (0.0625-64), chloramphenicol (0.125-256), clindamycin (0.016-32), ciprofloxacin (0.125-256), erythromycin (0.016-32), kanamycin (0.063-128), rifampicin (0.031-64), penicillin G (0.0625-64), streptomycin (0.125-256), tetracycline (0.125-256) and vancomycin (0.125-256). A two-fold dilution of each antibiotics, which were prepared in a 96-well microtiter plate by using Muller-Hilton broth (Hi-media, India) (final volume was 100 µl). Then, 100 µl culture broths of tested isolates

were inoculated into a microtiter plate (a final concentration was 10^5 CFU/ml) and incubated at 30°C for 24 h. After 24 h incubation, a growth inhibition of CNS was determined by turbidity at optical density ($OD_{600\ nm}$) using a microplate reader (BioTek®, USA). Minimal inhibitory concentrations (MICs) values of each antibiotic were evaluated as the lowest concentration of the sample tested that could prohibit bacterial growth at an inhibition rate higher than 90%. Interpretation for susceptibility status was evaluated in CNS according to the recommended standards for *Staphylococcus* species in EUCAST (2018). This study was performed in triplicate experiments and conducted two times on separate days using fresh strain culture.

5.3.2.3 Hemolytic activity test

The hemolysis detection is trait safety aspects to assess CNS isolates to be used as starter in fermented foods. The hemolytic activity was determined on blood agar containing 5% (v/v) sterile defibrinated human blood (Songklanakarin hospital, Thailand) in tryptic soy agar (TSA, Hi-media, India) through clear halo zone after incubation at 37°C for 24 h. The active CNS isolates were streaked on blood agar and then incubated at 37°C for 24 and 48 h (Zell *et al*., 2008). *S. aureus* DMST 8840 and *S. aureus* MRSA3 were used as controls for the hemolytic analysis. The hemolytic activity was examined by the appearance of clear halo formation (β-hamolysis), greenish zone (α-hemolysis) or absence of any zone (γ-hemolysis) around the colonies on blood agar.

5.3.2.4 Biogenic amines production analysis

The overnight culture of selected strain K21S22 was inoculated into 10 ml of TSB supplemented with 0.5% (w/v) of four biogenic amine precursors. The precursors were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany): L-histidine monohydrochloride, L-tyrosine disodium salt, L-lysine monohydrochloride and L-ornithine hydrochloride. After cultivation at 30°C for 7 days, cell-free supernatant (CFS) was collected by centrifugation and then analyzed by thin-layer chromatography (TLC) for biogenic amine production as described by (Saelao *et al*., 2017). Additionally, the presence of genes encoding biogenic amines production were amplified by PCR technique with specific primer sets as described by de las Rivas *et al*. (2006) (Table 14). PCR amplified products were demonstrated at 1.5% agarose gel electrophoresis.

Table 14 Primer set amplified encoding gene relating the biofilm formation, staphylococcal enterotoxins and biogenic amines production.

Target genes	Primers	Sequence 5´ to 3´	Tm (°C)	Target size (bp)	References
icaA	ica4f	TGG-GAT-ACT-GAY-AAT-GAT-TAC	55	568	Møretrø *et al*. (2003)
	ica2r	CCT-CTG-TCT-GGG-CTT-GAC-CAT-G			
sea	SEA-f	CCT-TTG-GAA-ACG-GTT-AAA-ACG	54	126	Moura *et al*. (2012)
	SEA-r	CTG-AAC-CTT-CCC-ATC-AAA-AAC			
seb	SEB-f	GGT-ACT-CTA-TAA-GTG-CCT-GC	55	475	Moura *et al*. (2012)
	SEB-r	TTC-GCA-TCA-AAC-TGA-CAA-ACG			
sec	SEC-f	AGA-ACT-AGA-CAT-AAA-AGC-TAG-G	55	267	Moura *et al*. (2012)
	SEC-r	TCA-AAA-TCG-GAT-TAA-CAT-TAT-CC			
sed	SED-f	TTT-GGT-AAT-ATC-TCC-TTT-AAA-CG	54	309	Moura *et al*. (2012)
	SED-r	CTA-TAT-CTT-ATA-GGG-TAA-ACA-TC			
see	SEE-f	CCT-ATA-GAT-AAA-GTT-AAA-ACA-AGC	53	173	Moura *et al*. (2012)
	SEE-r	TAA-CTT-ACC-GTG-GAC-CCT-TC			
hdc	HIS-f	GGN-ATN-GTN-WSN-TAY-GAY-MGN-GCN-GA	53	372	de las Rivas *et al*. (2006)
	HIS-r	ATN-GCD-ATN-GCN-SWC-CAN-ACN-CCR-TA			
odc	PUT1-f	TWY-MAY-GCN-GAY-AAR-ACN-TAY-YYT-GT	53	1440	de las Rivas *et al*. (2006)
	PUT1-r	ACR-CAN-AGN-ACN-CCN-GGN-GGR-TAN-GG			
ldc	CAD2-f	CAY-RTN-CCN-GGN-CAY-AA	53	1185	de las Rivas *et al*. (2006)
	CAD2-r	GGD-ATN-CCN-GGN-GGR-TA			
tdc	TDC-f	TGG-YTN-GTN-CCN-CAR-ACN-AAR-CAY-TA	53	825	de las Rivas *et al*. (2006)
	TDC-r	ACR-TAR-TCN-ACC-ATR-TTR-AAR-TCN-GG			

5.3.2.5 Detection of Staphylococcus enterotoxins (SEs) by PCR

The PCR technique can be useful for assessing the occurrence of SEs genes involving enterotoxin production in selected CNS strain before used as starter culture in fermented foods (Blaiotta *et al*., 2004). For the detection of SEs genes 5 different specific primer sets were previously described by Moura *et al*. (2012) (Table 14). PCR amplifications were performed in a total volume of 25 µl volume containing 2.5 µl of 2 µM of each primer, 12.5 µl of DreamTaq™ Green PCR Master Mix, 2 µl of DNA template and adjusted volume with DNase free water. Amplification program of these genes were performed in a thermocycler (Techne, Bibby Scientific, Staffordshire, UK) as described in Table 14. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel.

5.3.3 Technological properties

5.3.3.1 Effect of pH and NaCl on microbial growth

The starter candidate *S. carnosus* K21S22 was tested for growth capability at difference pH and NaCl concentrations followed the method described by Cachaldora *et al*. (2013) and Jeong *et al*. (2016) with some modification. The effect of pH on bacterial growth was evaluated in TSB broth adjusted to pH values of 4.0, 4.5, 4.8, 5, 5.5 and 6.0 by 6 N HCl. In additional, the ability of salt tolerance of this strain was determined in TSB broth supplemented with 5-15% NaCl. One hundred microliter of an overnight culture was inoculated into 10 ml of the different media described above and growth was evaluated by spreading on tested media after incubation at 30°C for 48 h.

5.3.3.2 Enzyme activity determination

Proteolytic and lipolytic activity of *S. carnosus* K21S22 were determined by caseinate and tributyrin agar assays (Cariolato *et al*., 2008; Bonomo *et al*., 2009; Landeta *et al*., 2013). This strain was grown in TSB broth and then 10 µl of each culture broth was dropped on the caseinate agar (1%, w/v sodium caseinate, Sigma-Aldrich, Germany) and tributyrin agar (1% w/v, tributyryl glycerol, Sigma-Aldrich, Germany) and incubated at 30°C for 72 h. Halo around the colonies indicated that it could hydrolyze the protein and lipid present in the medium. In addition, the activity of nitrate reductase was measured as previously described by Miralles *et al*. (1996) with slightly modifications. Overnight culture of selected strain was transferred to YT agar (0.5% yeast extract, 1% tryptone, 1.5% agar, pH 7.0) supplemented with 0.1% KNO_3 and incubated at 30°C for 20 h. After incubation, the plates were poured with a solution mixture comprised of the equal amounts of NIT1 (0.8% (w/v) sulfanilic acid in 5 N acetic acid) and NIT2 (0.6% (w/v) N, N-dimethyl-1-naphthylamine in 5 N acetic acid) for detecting nitrite. Red halo around bacterial colonies indicated that CNS strain exhibited the activity of nitrate reductase.

5.3.4 Role of autochthonous starter culture in the microbiological and sensory quality of Thai traditional fermented shrimp (*kung-som*)

5.3.4.1 Preparation of the starter culture

For the preparation of starter culture, 100 µl of glycerol stock of nisin Z-producing *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 were inoculated in 10 ml of

M17 and TSB broth, respectively. After incubation at 30°C for 24 h, 1 ml of the active culture was transferred to 30 ml of appropriate medium and incubated at 30°C for 24 h. Cells were harvested by centrifugation (10,300×g) for 15 min at 4°C and washed twice with sterile saline (0.85%). Finally, the cell pellets were adjusted to a density of 10^9 CFU/ml with sterile saline for used as a starter culture in *kung-som* production.

5.3.4.2 *Kung-som* preparation

Small shrimp were bought in Songkhla Province, Thailand. Samples were kept on ice and transported in ice pack to the Prince of Songkla University. Upon arrival, samples were immediately washed three times with water and divided into two equal portions. The original formula of *kung-som* consisted of 1 kg shrimp, 75 g NaCl, 300 g sugar and 250 ml water. The mixtures were transferred to glass jar and tightly covered with lid. *Kung-som* was performed by four different treatment: (1) without a starter (control); (2) with only the inoculation by nisin Z producing *Lc. lactis* KTH0-1S as starter culture (final concentration 10^7 CFU/g of samples); (3) with only the inoculation by *S. carnosus* K21S22 as starter culture (final concentration 10^7 CFU/g of samples); (4) combine with the inoculation by *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter culture (final concentration 10^7 CFU/g of samples). During fermentation, *kung-som* was sampled at the beginning of the fermentation and every 1, 2, 3, 5, 7, 10, 13, 16 and 20 days of fermentation. The physicochemical properties, microbiological changes and the volatile compound of finished *kung-som* products were determined by using HS-SPME technique in section 5.3.4.5

5.3.4.3 Physicochemical properties

The pH value of sample was determined by a pH meter (STARTER 2100, OHAUS, USA). For total acidity was measured by the titrimetric method, each sample (5 ml) was titrated against 0.1 M NaOH using phenolphthalein as an indicator. Total acidity was calculated as the equivalent lactic acid and expressed as percent of lactic acid according to Association of Official Analytical Chemists (AOAC, 2002).

5.3.4.4 Microbiological properties

Twenty-five grams of each sample was aseptically weighted in a sterile stomacher bag. Subsequently, samples were homogenized with 225 ml of 0.1% (w/v) sterile peptone water (Hi-Media, India). After mixing at 230 rpm for 1.30 min in a stomacher machine, the serial 10-fold dilutions were prepared from this homogenate

using peptone water (0.1% (w/v) peptone). The diluted samples (100 µl) were spread in triplicate on different growth media. Total viable count (TVC) was enumerated on plate count agar (PCA; Hi-Media, India) and incubated at 30°C for 48 h. The lactococci and lactobacilli counts were plated on M17 and MRS agar and incubated at 30°C for 24-48 h under anaerobic condition, respectively. Enterobacteriaceae and staphylococci were determined on violet red bile agar (VRBA; Hi-Media) and mannitol salt agar (MSA; Hi-Media) after an incubation at 37°C for 24-48 h, respectively. The microbiological data are expressed as logarithms of colony forming units per gram of sample (log CFU/g). Additionally, *S. aureus* enumeration was performed using a 5-tubes Most Probable Number (MPN) method as described by Bannett and Lancette (2001).

The microbiological safety of finished *kung-som* products was tested following to the recommendation of Thai Community Product Standard (TCPS, 2014). *Bacillus cereus* (less than 10^3 CFU/g), *Clostridium perfringens* (less than 10^3 CFU/g), *Escherichia coli* (less than 3 MPN/g), *S. aureus* (less than 10^2 CFU/g) and *Salmonella* sp. (not detected in 25 g of sample), yeast and molds (less than 10^3 CFU/g) were enumerated according to the Bacteriological Analytical Manual (BAM, 1998).

5.3.4.5 Volatile compounds extraction by headspace solid-phase micro-extraction (HS-SPME)

The volatile compounds were extracted by HS-SPME as described by Di Cagno *et al*. (2008) with some modification. Three grams of minced *kung-som* samples were placed into a 20 ml headspace vial and sealed with a polytetrafluoroethylene (PTFE)-faced silicone septum. The vials were incubated at 60°C in during 30 min to obtain equilibration. Then, a SPME fiber (75 µm, carboxen/polydimethylsiloxane (CAR/PDMS; Supelco, Sigma-Aldrich, Darmstad, Germany) was exposed to the headspace while maintaining the sample. It was immediately followed by desorption of the fiber in the gas chromatography injector for 10 min at 240°C.

5.3.4.6 Gas chromatography mass spectrometry (GC-MS) analysis

The GC-MS analyzes were performed using a TRACE™ Ultra GC system coupled with an ISQ™ Single Quadrupole mass spectrograph (Thermo Scientific, MA, USA). The volatile compounds were separated in a AT-WAXms

capillary column (30 m × 0.25 mm × 0.25 μm, Heliflex®, Croydon, England). GC-MS conditions were as follows: spilt less injection with 1 min delay; carrier gas was helium at constant flow rate of 1.0 ml/min. The GC oven program was held at 38°C for 13 min, raised to 110°C at a rate 3°C/min, raised to 150°C at 4°C/min, raised 210°C at 10°C/min and then held at 210°C for 5 min. The temperature of injector, transfer line and ion source were all at 240°C. The mass spectrometry obtained on electron ionization (EI) mode at 70 eV and scanning rang (*m/z*) was 29-400 amu with 0.2 s interval. Volatile compounds were identified comparing their mass spectra of those from the library in National Institute of Standards and Technology (NIST) and Wiley 9.0 mass spectra library databases. The approximate quantities of volatile profile composition were expressed as relative percentage of each single peak area with respect to the total peak area.

5.3.4.7 Sensory evaluation

Kung-som produced by with or without the inoculation of starter culture was evaluated by 30 panelists (10 males and 20 females with an age between 25 and 50 years), they were selected from the staffs and graduate students in Faculty of Agro-Industry, Prince of Songkla University. All panelists were familiar with consuming *kung-som* since at least three years. Each *kung-som* sample was marked with three-digit numbers, then placed in a plastic cup and covered with lids prior to serve for the sensory testing. Sensory assessment was performed using a point 9-hedonic scale in which 9 was the highest score and 0 was none (Dajanta *et al*., 2011). Panelists were asked to evaluate appearance, color, flavor, taste, texture and overall acceptance. Before starting sensory testing and between sample assessments, panelists were instructed to rinse their mouth with water.

5.3.5.8 Statistical analysis

The data in this study was subjected to one-way analysis of variance (ANOVA) with Duncan's multiple range tests to identify the significant difference at $p<0.05$ using the Statistical Package for the Social Science (SPSS 16.0 for windows, SPSS Inc., IL, USA).

5.4 Results and Discussion

5.4.1 Prevalence of characteristic involved in safety of CNS isolated from *kung-som*

The safety evaluation of microorganism in food or feed materials for QPS status according to EFSA (2005) standard requiring the safety evidence such as antibiotic susceptibility and no virulence factors (EFSA, 2013). However, the typical virulence factors for CNS have not yet been clearly determined. Several studies reported the opportunistic infection, toxigenicity and antibiotic resistance as well as presence of transferable antibiotic resistance genes of CNS strains (Zell *et al*., 2008; Moura *et al*., 2012; Chajecka-Wierzchowska *et al*., 2015). In this study, the safety assessments of CNS isolated from Thai fermented shrimp (*kung-som*) were tested for the virulence factors to be referred as previously reported by Marty *et al*. (2012), Jeong *et al*. (2014) and (2016).

The biofilm formation is undesirable for hygiene and safety reason because pathogenic or spoilage bacteria was capable to adhere to food product surface that detected by spectrophotometric and biofilm encoding gene. In the present study, the 32 isolates (69%) of CNS exhibited moderate activity of biofilm formation, 4 isolates (9%) of CNS showed weak activity and 10 strains (22%) as non-biofilm forming as determined by spectrophotometry method (Table 15). Landeta *et al*. (2013) reported that 4% of CNS strains isolated from Spanish meat product were able to produce biofilm whereas Leroy *et al*. (2009) and Jeong *et al*. (2014) reported that 30% and 74% of CNS strains could be formed biofilm. This ability is a fundamental survival strategy for most prokaryotic organisms, forming biofilm of CNS depended on strain-dependent characteristic and the external factors such as stress conditions (Møretrø *et al*., 2003; Landeta *et al*., 2007b; Jeong *et al*., 2014). It has been reported that pathogenic can be alter gene expression to form biofilm in competitive condition or protect against antibiotic treatment. However, the formation does not directly present virulence (Bizzini *et al*., 2006). For safety aspect, the presence of the *icaA* gene involving biofilm forming has been checked. Accordingly, the result obtained from PCR assay for detection of *icaA* gene revealed the negative effect of 10 CNS isolates (Fig. 30). All of CNS non-biofilm formation isolates were identified to species level by sequencing of 16S rRNA gene. They belonged to the species (99% similarity) of *Staphylococcus*

carnosus (1 strain), *S. condimenti* (5 strains), *S. piscifermentans* (3 strains) *and S. xylosus* (1 strain). Many publications reported that CNS strains such as *S. condimenti, S. carnosus*, *S. equorum*, *S. piscifermentans*, *S. succinus* and *S. xylosus* are frequently isolated from fermented food and may therefore have the potential for future application as starter cultures in fermented products.

Table 15 Biofilm formation of CNS isolates from Thai traditional fermented shrimp (*kung-som*).

Isolates	Biofilm formation[a] A_{595}	Isolates	Biofilm formation[a] A_{595}
S. aureus DMST 8840	+++	K16S1	++
K0S8	+++	K16S2	-
K2S6	-	K16S3	+++
K2S8	+	K16S4	+
K2S12	-	K21S3	+
K4S5	+++	K21S4	+++
K4S6	++	K21S5	-
K4S7	+++	K21S6	+++
K5S15	-	K21S7	+++
K8S1	+++	K21S8	+
K8S2	++	K21S9	-
K8S3	+++	K21S10	+++
K8S5	-	K21S11	-
K8S6	+++	K21S12	+++
K8S21	-	K21S13	+++
K10S2	++	K21S14	+++
K10S3	++	K21S15	+
K10S4	+++	K21S16	+++
K13S2	++	K21S17	+
K13S3	++	K21S18	+++
K13S4	+	K21S19	++
K13S7	+++	K21S20	+++
K13S8	++	K21S22	-
K13S9	+++		

[a]Biofilm production. spectrophotometric method: (−) $A_{595} \leq 1$; (+) $A_{595} \leq 2$; (++) $A_{595} \leq 3$; (+++) $A_{595} > 3$.

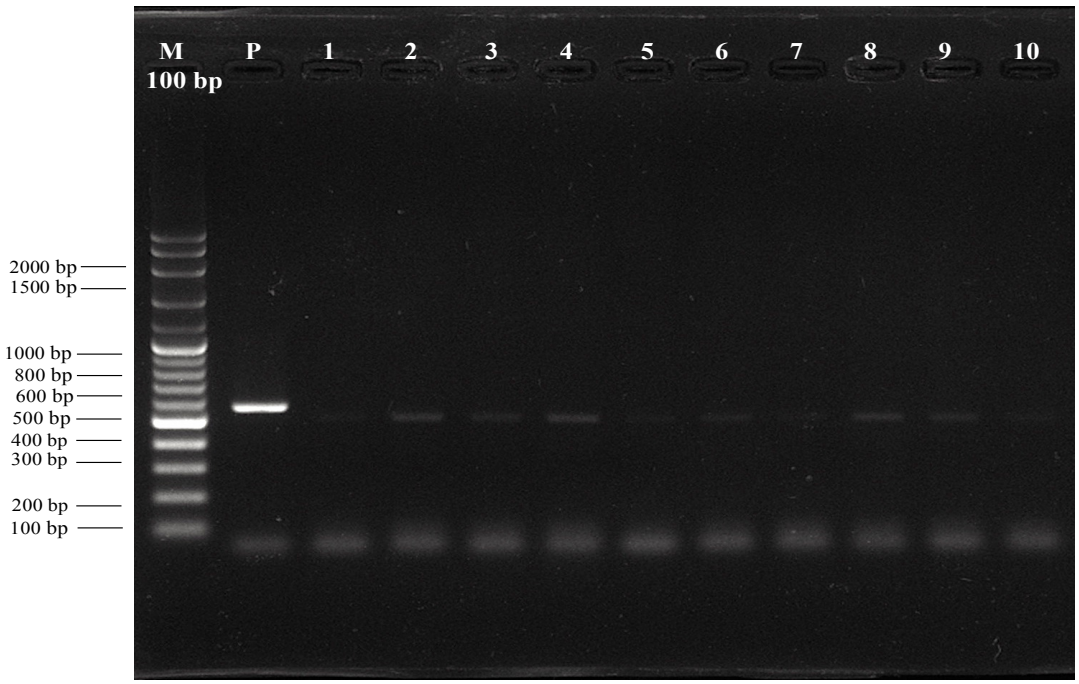

Figure 35 The presence of *icaA* gene involving the biofilm formation in CNS strains isolated from *kung-som* was detected by PCR using specific primers (expected size 568 bp). Lane P: *S. aureus* DMST 8840 (positive control); lane 1: *S. carnosus* K21S22; lane 2: *S. condiment* K8S5; lane 3: *S. condimenti* K16S2; lane 4: *S. condimenti* K21S5; lane 5: *S. condimenti* K21S9; lane 6: *S. condimenti* K21S11; lane 7: *S. piscifermentans* K2S12; lane 8: *S. piscifermentans* K5S15; lane 9: *S. piscifermentans* K8S21; lane 10: *S. xylosus* K2S6 and lane M: 100 bp DNA molecular weight marker (Vivantis, Malaysia).

According to the intensive use of antibiotics in public health and animal husbandry, resistance to antibiotics could occur in CNS strains isolated from foods (Marty *et al*., 2012; Chajecka-Wierzchowska *et al*., 2015). Therefore, the antibiotic resistance properties in CNS isolates is safety aspect before using bacterial strains to be used as starter culture in fermented foods. Ten non-biofilm forming CNS strains were selected for further antibiotics susceptibility testing by microdilution assay. If the strains present the MIC_{90} value higher than MIC breakpoint established by EUCAST (2018), they are considered as antibiotic resistance.

Our finding demonstrated that *S. carnosus* K21S22 and *S. conditmenti* K8S5, K16S2, K21S5 and K21S11 were sensitive to all tested antibiotics (Table 16). This is in agreement with previous reports that *S. carnosus* and *S. condimenti* species exhibit markedly susceptibility or less antibiotic resistance than other CNS strains (Resch *et al*., 2008; Seitter *et al*., 2011). However, other studies demonstrated that those strains of *S. carnosus* were resistant to cefotaxime, chloramphenicol, oxacillin, streptomycin and trimethoprim (Marty *et al*., 2012; Müller *et al*., 2016). Contrarily, most *S. piscifermentans* and *S. xylosus* were resistant to at least two antibiotics belonging to different antimicrobial classes (Table 16). This finding is in accordance with previous reports where an increasing resistance to multiple antibiotics has been reported in medical and food-related isolates (Seitter *et al*., 2011; Marty *et al*., 2012). As previous studies on antibiotic resistances of fermented foods associated CNS, resistance to penicillin (20-43%), ampicillin (15-31.9%), tetracycline (14-34.5%), erythromycin (3.2-32.4%) clindamycin (4.0-36.2%) were relatively high, while chloramphenicol (4.8-6%), rifampicin (0.6%) and streptomycin (5%) were presence in low frequencies (Marty *et al*., 2012; Jeong *et al*., 2014; Chajecka-Wierzchowska *et al*., 2015).

Accordingly, our result suggested that the strain-specific intrinsic resistance in CNS strains isolated from *kung-som* might be reflected the environment where these bacteria are found as well as exposure to antibacterial agents (Jeong *et al*., 2014; 2016). Therefore, the CNS strains sensitive to tested antibiotics were selected for starter candidates to eliminate the potential risk of antibiotic resistance gene lateral transfer.

Table 16 Biofilm formation and antibiotic susceptibility of CNS strains isolated from *kung-som*.

Species	**Biofilm**[a]		**Antibiotic susceptibility**[b]										
	SM	icaA	Am	Clo	Cli	Cip	Ery	Kan	Rif	PeG	Str	Tet	Van
S. aureus DMST 8840	+++	+	0.25	4	0.125	0.5	4	4	0.063	2	8	2	1
S. carnosus K21S22	-	-	0.125	4	0.063	<0.25	0.25	1	0.063	<0.063	4	0.25	1
S. condimenti K8S5	-	-	0.125	8	0.125	<0.25	0.25	1	0.063	0.063	4	0.25	1
S. condimenti K16S2	-	-	0.125	4	0.125	<0.25	0.25	1	0.063	0.063	4	0.25	1
S. condimenti K21S5	-	-	0.125	4	0.125	<0.25	0.25	1	0.063	0.063	4	0.25	1
S. condimenti K21S9	-	-	0.25	4	0.063	<0.25	0.25	2	0.063	0.125	16	0.25	1
S. condimenti K21S11	-	-	0.125	4	0.125	<0.25	0.25	1	0.063	0.063	4	0.25	1
S. piscifermentans K2S12	-	-	0.125	4	0.125	<0.25	0.25	8	0.125	<0.063	16	0.25	1
S. piscifermentans K5S15	-	-	0.125	4	0.125	<0.25	0.25	8	0.25	<0.063	16	0.25	1
S. piscifermentans K8S21	-	-	0.125	8	0.25	<0.25	0.5	8	0.063	<0.063	8	0.5	1
S. xylosus K2S6	-	-	1	16	64	0.5	>64	>128	2	2	128	>64	1
MIC breakpoint[c]			0.125	16	0.25	1	1	8	0.063	0.125	8	1	2

[a]Biofilm formation. Spectrophotometric method (SM): (−) $A_{595} \leq 1$; (+) $1 < A_{595} \leq 2$; (++) $2 < A_{595} \leq 3$; (+++) $A_{595} > 3$. icaA: PCR amplification of *icaA* gene involved biofilm production: (+) positive; (-) negative.

[b]Antibiotic resistance: ampicillin (Am); chloramphenicol (Clo); clindamycin (Cli); ciprofloxacin (Cip); erythromycin (Ery); kanamycin (Kan); rifampicin (Ri); penicillin G (PeG); streptomycin (Str); tetracycline (Tet); vancomycin (Van). R: resistant; S: sensitive.

[c]MIC breakpoint was established by EUCAST (2018).

Moreover, the hemolysin considered as virulence factor, therefore, the absence of hemolytic activity has been suggested as a selection criterion for starter cultures in the food and feed sector starter strains in food production (EFSA, 2006; Marty *et al*., 2012). Hemolysins are exotoxins that cause the lysis of erythrocytes by pore formation (α-hemolysis) or by degrading sphingomyelin (β-hemolysis) (Berube and Wardenburg, 2013). Phenotypic hemolytic activity testing has commonly been observed on sheep blood (SB) and human blood agar (HB) in CNS isolated from various foods including commercial meat starter cultures (Zell *et al*., 2008). Furthermore, PCR amplification of the previously known hemolysin genes discorded from results of phenotypic hemolytic activities was also reported by Marty *et al*. (2012).

Therefore, the phenotypic hemolytic activity testing is more practicable than the identification of their determinants for safety assessment. Six CNS strains which were sensitive to tested antibiotics as well as non-biofilm formation were selected for testing hemolytic activity on HB agar. Only strain *S. carnosus* K21S22 exhibited non-hemolytic activity on HB agar (Fig. 36), while positive hemolysis observed in five other CNS strains including *S. condimenti* (4 strains) and *S. piscifermentans* (1 strain) which were classified as β-hemolytic activity (weak activity). Zell *et al*. (2008) reported weak to moderate hemolytic activity in 86% of *S. equorum*, 83.9% of *S. xylosus*, 63.6% of *S. piscifermentans*, 50% of *S. condimenti*, 40% of *S. succinus* and 10.4% of *S. carnosus* in hemolytic activity assessment of 330 CNS isolates from various food and commercial starter cultures.

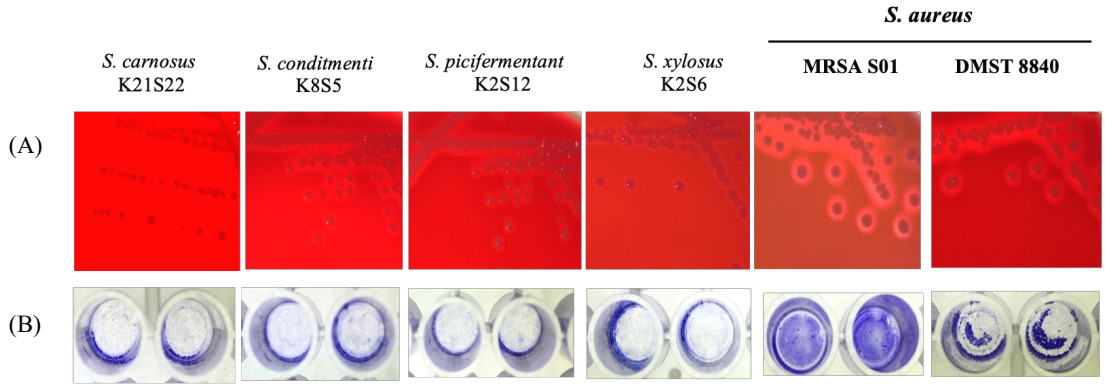

Figure 36 Hemolytic activity (A) and biofilm formation (B) of CNS strains isolated from *kung-som*. *S. aureus* DMST 8840 and MRSA S01 were used as the positive control for hemolytic activity and biofilm formation.

Staphylococcal intoxication, one of the main food-borne diseases, has been associated with coagulase-positive *S. aureus* and its production of staphylococcal enterotoxins (SEs). SEs are a wide array of toxins that cause various types of disease symptoms and that are heat stability and resistance toward most gastrointestinal proteolytic enzymes (Nema *et al*., 2007). *S. aureus* has been regarded the representative capable of SEs production. On the other hand, CNS have been considered to be non-pathogenic bacteria due to most CNS can establish a commensal relation with human and animals (Otto, 2010). However, previously report demonstrated that 45.7% of CNS strains isolated from various foods were able to produce enterotoxins (Zell *et al*., 2008). Previous reports revealed that SE genes can be spread among staphylococci strains and can be modified their capability to cause disease (Argudín *et al*., 2010; Jeong *et al*., 2014). Many publications successfully detected the SEs genes by using PCR amplification to confirm existence of these genes in CNS strains (Even *et al*., 2010; Moura *et al*., 2012; Fijałkowski *et al*., 2016). The most prevalent genes were *sea* (90%) and *seb* (70%) in CNS isolates (*S. saprophyticus*, *S. piscifermentans*, *S. sciuri* and *S. xylosus*) (Casaes Nunes *et al*., 2016). In the present study, our result revealed that *S. carnosus* K21S22 did not harbor SE genes (*sea*, *seb*, *sec*, *sed* and *see*) (Fig. 37). Other authors reported similar results and concluded that the presence of SEs genes is a strain dependent characteristic in CNS isolated from fermented foodstuffs (Blaiotta *et al*., 2004; Even *et al*., 2010; Jeong *et al*., 2014).

Among safety aspects associated to CNS (and other food-grade bacterial species), the non-biogenic amines (BAs) formation is a safety criterion with regard to apply CNS strains as a starter culture based on the QPS concept (EFSA, 2011a). BAs are originated through amino acid decarboxylation by microorganism in various fermented products, especially in protein-rich meat products, fish products and cheeses (Silla Santos, 1996). Major biogenic amines can be found in foods such as histamine, putrescine, cadaverine and tyramine. Tyramine and histamine are well known as dangerous compounds to human health and their leads to acute adverse effect (psychoactive and vasoactive properties). The food composition and bacterial growth are the factors which strongly effected on types and amounts of biogenic amines in fermented food (Carelli *et al*., 2007).

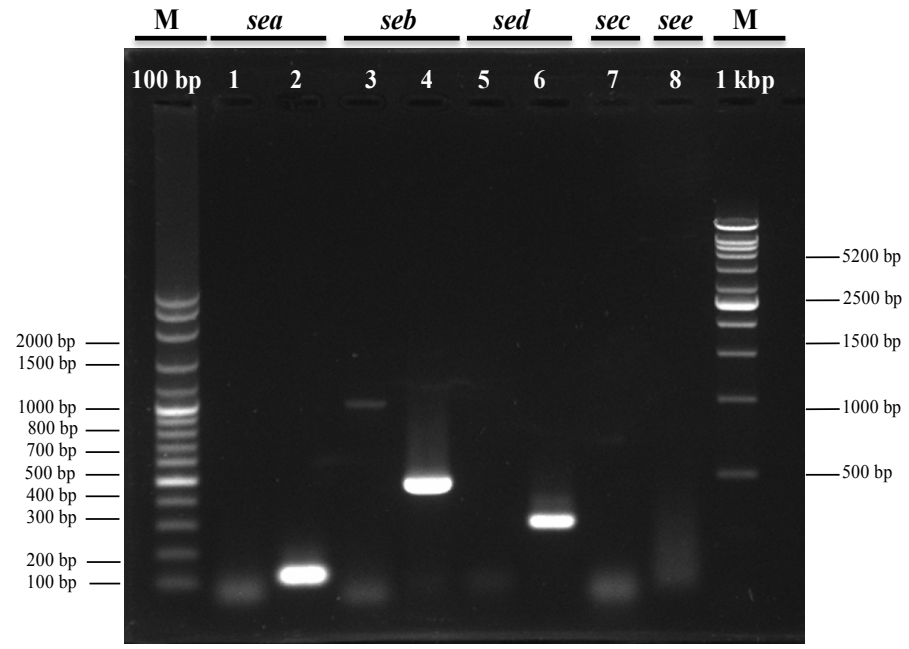

Figure 37 Amplification by PCR of SEs genes in *S. carnosus* K21S22: Lanes 1, 3, 5, 7 and 8: PCR products of corresponding genes obtained for *S. carnosus* K21S22; lane 2, 4 and 6: amplification of *sea* (*S. aureus* MRSA S03), *seb* (*S. aureus* MSSA S03) and *sed* (*S. aureus* MRSA S01) genes from positive strains and lane M: 100 bp and 1 kb DNA molecular weight marker.

The criterion of this work was to evaluate the ability of the BAs production for eliminating the potential safety hazards produced by CNS for used as starter cultures. *S. carnosus* K21S22 was tested *in vitro* conditions favoring BAs production. It was found that this strain did not produce the cadaverine, histamine, putrescine and tyramine (Fig. 38A). This finding is in accordance with Müller *et al.* (2016) who reported that *S. carnosus* did not form their biogenic amines. In agreement with literature, the production of cadaverine, histamine and putrescine could be rarely detected in *S. carnosus* strain (Martín *et al*., 2006; Seitter *et al*., 2011), but often in *S. equorum* (Jeong *et al*., 2014), *S. epidermidis* (Even *et al*., 2010), *S. lugdunensis* and *S. capitis* (histamine-producing strain) (Landeta *et al*., 2007b). In addition, it has been reported that 25% of *S. carnosus* strains isolated from dry-cured sausage were capable to generate the tyramine *in vitro* condition (Landeta *et al*., 2013).

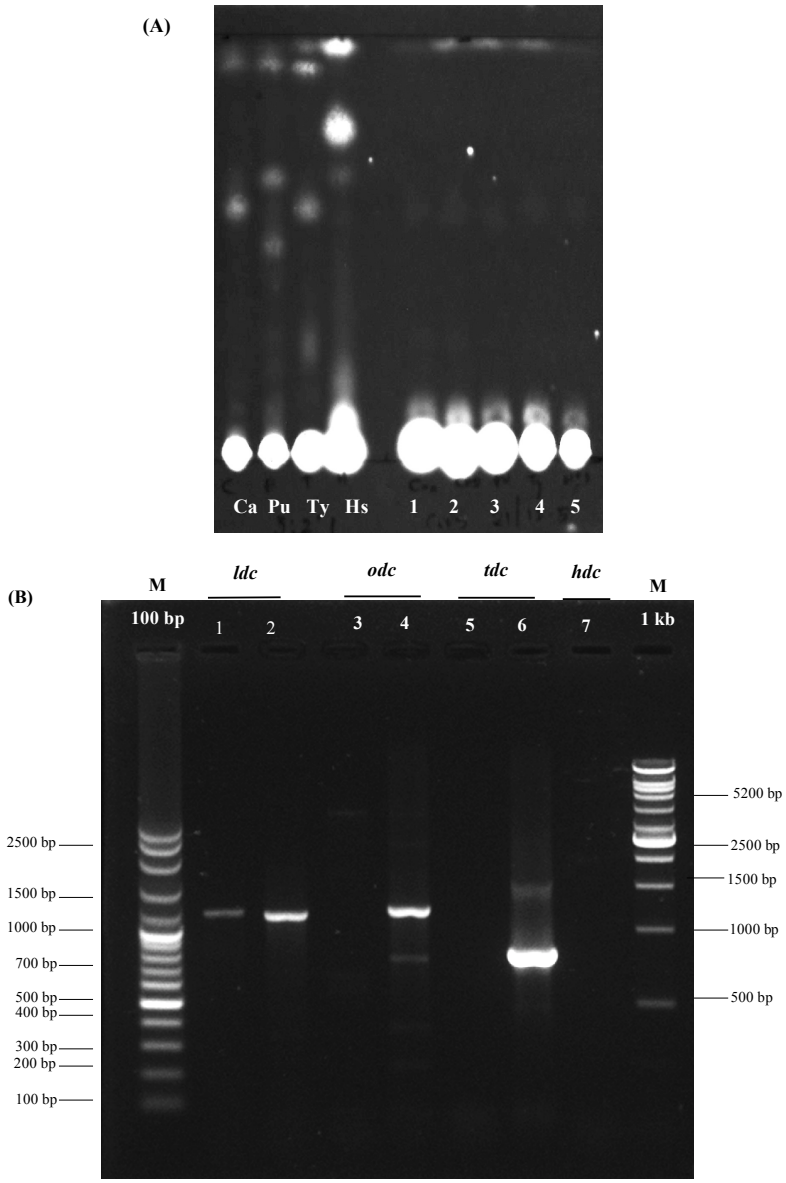

Figure 38 (A): The dansylated biogenic amines standards as separated by TLC: cadavarine (Ca), putrescine (Pu), tyramine (Ty), and histamine (Hs). Cell-free supernatant of *S. carnosus* K21S22 grown in TSB media containing: (1) without substrate, (2) 0.5% lysine, (3) 0.5% ornithine, (4) 0.5% tyrosine, and (5) 0.5% histidine were dansylated and separated on TLC plate. (B): The presence of amino acid decarboxylase genes in *S. carnosus* K21S22 was detected by PCR using specific primers. Lanes 1, 3, 5 and 7 amplification products of corresponding genes obtained for *S. carnosus* K21S22; lanes 2, 4, and 6 amplification products of corresponding genes obtained for positive controls. Lane M: 100 bp and 1 kb ladder DNA markers.

In order to correlate the BAs production with the presence of the corresponding decarboxylase genes, PCR assays for the detection of these genes was performed. Accordingly, the results obtained by PCR showed positive band corresponding to *ldc* gene (Fig. 38B). However, the size of this band differed from a positive control (Fig. 38B) as well as its nucleotide sequence was 99% similarity of 16S rDNA gene of *Staphylococcus* sp. (Appendix B, page 205), indicating that *S. carnosus* K21S22 did not harbor any amino acid decarboxylase genes (*hdc*, *ldc*, *odc* and *tdc* genes). From obtained results in the present study, we can conclude that the aminogenic capacity of *S. carnosus* strain is strain-specific and should therefore be tested individually for each strain with intended application in food. Our finding suggests that *S. carnosus* K21S22 was nonexistence of acquired risk factors. Accordingly, it would be safe to be used as starter culture regarding to the QPS concept.

5.4.2 Technological properties of starter candidates

CNS are considered positive impact on fermentation processes and the sensory characteristics of the products and frequently used as a starter culture in fermented meat, fermented sausage and fermented seafood (Marty *et al*., 2012; Landeta *et al*., 2013; Jeong *et al*., 2014). Not only safety aspects starters need also to have certain technological properties to be adopted for food fermentations. However, CNS strains have not been applied as starter culture in *kung-som* so far. Therefore, the requirements of technological properties of a starter culture for *kung-som* fermentation have not yet been reported. Several researchers demonstrated that CNS isolates were able to grow under the difference of pH values and salt concentrations, which are limiting factors in the starter cultures selection (Bonomo *et al*., 2009; Zaman *et al*., 2014). In the present study, *S. carnosus* K21S22 grew well at pH 5 to 7 (~7.15 log CFU/ml) on tested media, but did not grow at pH lower than pH 4.5 (Table 17). This is in agreement with result of Casaburi *et al*. (2005). The obtained result showed the salt concentration (5-12% NaCl at pH 5) did not significantly affect growth of strain K21S22 (cell viability >7.0 log CFU/ml). Consequently, salt tolerance of at least 7% (pH 5) in *S. carnosus* K21S22 was a good characteristic to be used this strain as a starter culture for *kung-som* production. The proteolytic and lipolytic activities in CNS significantly generated to the low molecular weight substances (peptides, aldehydes, free fatty acids, etc.) for developing the favor compounds (Leroy *et al*., 2006). Nevertheless, little is known

regarding the relationship between these sensory properties and microorganism growth in *kung-som* fermentation. When considering the high protein and lipid content of *kung-som*, the protease and lipase activities of CNS that function during fermentation could contribute to the aroma quality of *kung-som*. Related to proteolytic and lipolytic activities, the agar plate assay was used in this study, *S. carnosus* K21S22 presented protease and lipase activities on tested medium (Table 17).

Table 17 Morphological and technological characteristics of *S. carnosus* K21S22.

Characteristic	***S. carnosus* K21S22**
Colony size and shape	white colony, cocci
Gram staining	positive
Catalase test	positive
Proteolytic activity	positive (radius 5.0 mm)
Lipolytic activity	positive (radius 5.0 mm)
Nitrate reductase activity	positive (radius 3.5 mm)
Growth in TSB with (pH) (log CFU/ml)	
4.0	nd.
4.5	4.28 ± 0.1^{c}
4.8	6.32 ± 0.1^{b}
5.0	7.15 ± 0.1^{a}
5.5	7.18 ± 0.1^{a}
6.0	7.19 ± 0.3^{a}
7.0 (control)	7.20 ± 0.3^{a}
Growth in TSB with (NaCl at pH 5) (log CFU/ml)	
0.5% (control)	7.19 ± 0.1^{a}
5%	7.20 ± 0.1^{a}
7%	7.16 ± 0.2^{a}
10%	7.16 ± 0.2^{a}
12.5%	6.88 ± 0.1^{b}
15%	No growth

[a-c]Different superscript within a column represent significant differences ($p<0.05$).
nd: non-detectable.

The obtained results in the previous study showed that the proteolytic and lipolytic activities of CNS were variability depending on strain-specific (Landeta *et al*., 2013; Jeong *et al*., 2016). Additionally, *S. carnosus* K21S22 has nitrate reductase capacity on agar plate assay (Table 17). This activity has frequently been found in strains of *S. carnosus*, *S. equorum* and *S. xylosus*. Its ability could be generated a stable color in fermented meats (Talon *et al*. 2008; Sánchez Mainar and Leroy, 2015; Jeong *et al*., 2016; Sánchez Mainar *et al*., 2017). Although, the nitrate reductase activity did not specifiable for *kung-som* starter culture, its activity will be applied in various fermented foods. Our results suggested that *S. carnosus* K21S22 was safe and produce many enzymes including protease, lipase and nitrate reductase. Moreover, this strain was able to grow in high salt concentration and acid condition. Therefore, *S. carnosus* K21S22 has potential to be developed as starter culture incorporating with nisin Z-producing *Lc. lactis* KTH0-1S for improving quality, sensory and safety in *kung-som* product.

5.4.4 Effect of autochthonous nisin Z-producing *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 during *kung-som* fermentation

5.4.4.1 Chemical and microbiological changes during *kung-som* fermentation and finished *kung-som* product

The chemical and microbiological changes were investigated during *kung-som* fermentation for the different treatments. The initial pH of all batches of *kung-som* was pH 6.9 (Fig. 39). During fermentation, pH of *kung-som* inoculated with *Lc. lactis* KTH0-1S as starter culture (LS and LCS batches) gradually decreased to <4.6 within the 13 days, and then remained stable at ~4.3 throughout the fermentation time (Fig. 39). Contrary, the pH value of the control (without starter) and *kung-som* inoculated with only strain *S. carnosus* K21S22 as starter culture (C and CS batches) gradually decreased to 4.6 only within 16 days. In addition, the lactic acid content of inoculated *kung-som* batches (LS and LCS sets) were significantly higher than in the two control sets. The detected amount of lactic acid in LS and LCS batches reached values of 1.9-2.2% within 10 days, whereas it reached a comparable concentration of ~2.0% at the end of the fermentation (day 20) for the control and CS batches determined (Fig. 39).

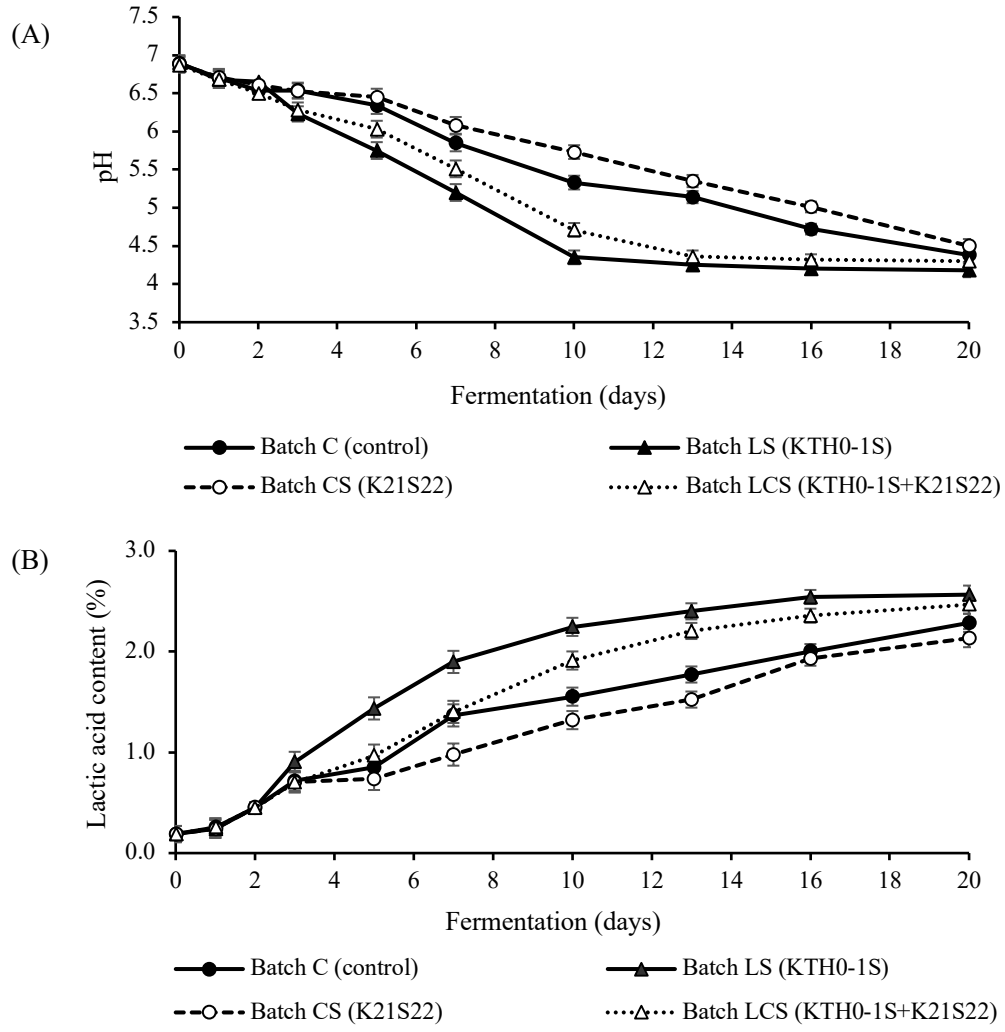

Figure 39 Changes of pH (A) and total acidity as lactic acid (B) during *kung-som* fermentation. Bars represent the standard deviation from three determinations.

Changes of microbial count during *kung-som* fermentation are shown in Table 18. The initial population of LAB, CNS and TVC in the inoculated *kung-som* was significantly (p<0.05) higher than in the control batch (Fig. 40). LAB count of inoculated *kung-som* in LS and LCS batches showed significantly (p<0.05) higher than CS and control batches, reaching of levels of 9.0, 9.1 7.2 and 8.5 log CFU/g at 5 days of fermentation, respectively. Then LAB, especially lactococci count slightly decreased to reach level below 4.0 CFU/g for all batches at the end of fermentation (Fig. 40). This result revealed that LAB as starter culture was able to adapt and grow well in the *kung-som* environment. LAB as starter culture contribute to desirable texture, color and

flavor and have a positive effect on the hygiene properties of the product, inhibiting pathogenic and spoilage bacteria by acidification or by the production of antimicrobials (Villani *et al*., 2007; Lorenzo *et al*., 2014; Sanchart *et al*., 2017). While initial of CNS counts were 4.4 and 4.5 log CFU/g in *kung-som* inoculated with only *Lc. lactis* KTH0-1S (LS batch) and control treatment (C batch) which showed significantly (p<0.05) lower than *kung-som* produced by inoculation of *S. carnosus* K21S22 as starter culture (CS and LCS batches) (Fig. 40). On the 10th day of fermentation, CNS numbers increased to reach 9.4, 8.9, 5.6 and 7.1 log CFU/g for CS, LCS, LS and C batches, respectively. The presence of high amounts of CNS in batch LCS during fermentation suggested the good competitiveness of staphylococci due to they well adapted under the intensive growth of LAB associated with a decrease of pH as reported by previous works (Casquete *et al*., 2012; Tabanelli *et al*., 2012).

Additionally. TVC counts gradually increased to 9.0 log CFU/g on 5 days of fermentation, whereas the inoculated kung-som with nisin Z-producing *Lc. lactis* KTH0-1S (LS and LCS sets) shopwed significantly (p<0.05) lower than TVC numbers (Fig. 40). A slightly decrease of Enterobacteriaceae observed in the spontaous *kung-som*, while the dratic reduction occurred becoming undetecable in the inoculated *kung-som* (LS and LCS batches) after 7 and 10 days of fermentation, respectively. These results are in agreement with those of Sanchart *et al*. (2017) found that Enterobacteriaceae counts were steadily decreasing due to the acidifying activity of starter culture used in *kung-som*. Moreover, the initial counts on *S. aureus* in all batches increased within the first 5 days to reach >540 MPN/g, and the continually decreased until non-detectable after 10 days of fermentation for *kung-som* with and without a starter (Table 18), respectively. *S. aureus* reduction might result in the contribution of organic acid and bacteriocin produced by bacteriocinogenic strain (Gao *et al*., 2014a; Wang *et al*., 2015). Our results are in accordance with previous reports in which the bacteriocinogenic strains were used as starter culture in fermented products that could inhibit Enterobacteriaceae and *S. aureus* growth during fermentation process (Bover-Cid *et al*., 2001a; Lauková *et al*., 2010b; Tosukhowong *et al*., 2011).

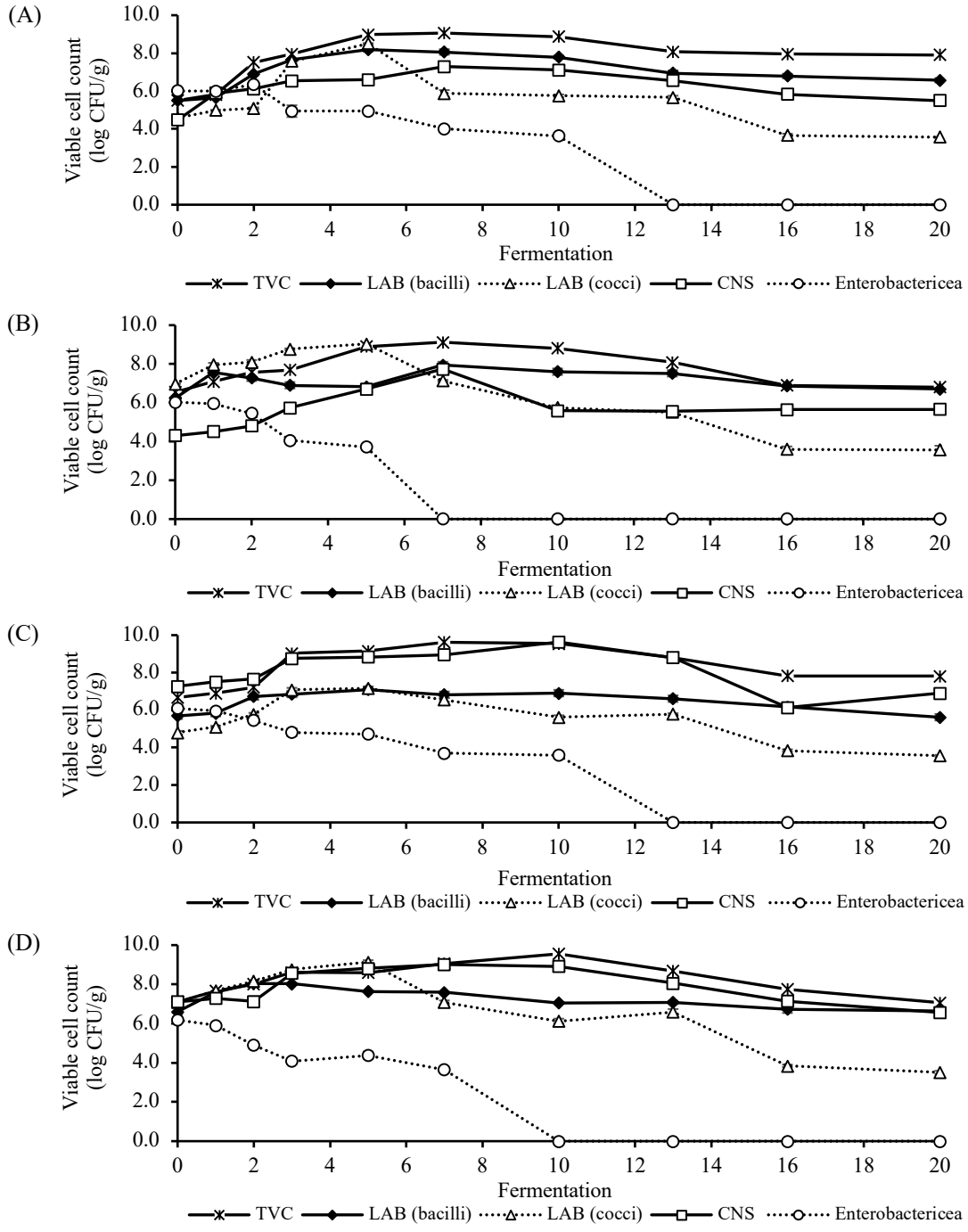

Figure 40 Changes of microbial populations of spontaneous *kung-som* (A), inoculated *kung-som* with *Lc. lactis* KTH0-1S as a starter culture (B), inoculated *kung-som* with *S. carnosus* K21S22 as a starter culture (C) and inoculated *kung-som* with mixed of *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter cultures during fermentation.

Table 18 *S. aureus* counts (MPN/g) in *kung-som* produced by with and without starter cultures inoculation.

Microbial counts	Batch[A]	Fermentation (days)									
		0	1	2	3	5	7	10	13	16	20
S. aureus (MPN/g)	C	11[a]	11[a]	17[a]	1600[b]	>1600[a]	540[a]	17[a]	nd	nd	nd
	LS	7.8[b]	11[a]	17[a]	540[d]	240[d]	11[b]	nd	nd	nd	nd
	CS	7.8[b]	11[a]	17[a]	>1600[a]	>1600[a]	540[a]	14[b]	nd	nd	nd
	LCS	11[a]	11[a]	14[b]	920[c]	540[c]	17[c]	nd	nd	nd	nd

[a-d] Different superscripts within a column represent significant differences (p<0.05); nd: non-detectable.

[A] Batch C: natural fermented *kung-som*, Batch LS: *kung-som* inoculated with only nisin Z-producing *Lc. lactis* KTH0-1S as a starter culture, Batch CS: *kung-som* inoculated with only *S. carnosus* K21S22 as a starter culture and Batch LCS: *kung-som* inoculated with mixed culture of *Lc. lactis* KTH0-1S and *S. carnosus* K21S22.

The physicochemical properties and microbiological safety of finished *kung-som* (4 treatments) were determined prior to sensory evaluation. The pH value of all finished products was lower than 4.6. Total acidity as lactic acid of inoculated batches with *Lc. lactis* KTH0-1S (~2.5%) was significantly (p<0.05) higher than that of products with only *S. carnosus* K21S22 as starter culture and without starter culture (~2%). Microbiological safety of *kung-som* was investigated following to the recommendation of Thai Community Product Standard (TCPS, 2014). *B. cereus*, *C. perfringens*, *E. coli*, *Salmonella* sp. and *S. aureus* did not detect in any samples, while yeast and molds were detectable level less than 2.0 log CFU/g. The obtained results suggest that all *kung-som* samples are safe for consumption according to TCPS (2014). These results showed that the addition of *Lc. lactis* KTH0-1S and mixed strains (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) as starter culture significantly reduced (p<0.05) the pathogenic and spoilage bacteria faster than control treatments during *kung-som* fermentation.

5.4.4.2 The volatile profiles of *kung-som* products inoculated with bacteriocinogenic *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter culture

Twenty-eight volatile compounds from *kung-som* produced with or without *Lc. lactis* KTH-1S or/and *S. carnosus* K21S22 as starter culture were identified by using HS-SPME method coupled with GC-MS analysis (Table 19) (Fig. 41-44). *Kung-som* inoculated with *Lc. lactis* KTH0-1S or/and *S. carnosus* K21S22 were significantly difference (p<0.05) in comparison with control treatment. The volatile groups of all inoculated *kung-som* had value of volatile compounds higher (p<0.05)

than control treatment. The volatile compounds obtained from all *kung-som* treatments can be grouped as esters (8), acids (7), alcohols (6) and others (4) (Table 19). Several researchers reported that the formation of volatile compounds originated from carbohydrate fermentation, lipid oxidation, amino acid degradation as well as microbial metabolism (Bourdat-Deschamps *et al*., 2004; Beck, 2005; dos Santos *et al*., 2015).

Acids were the most abundant volatile compounds from all *kung-som* samples, representing from 38.64-75.27% of the total relative peak area (Table 19). In particular, propanoic, butanoic, pentanoic, 2-methylpropanoic, 2-methylbutanoic and 3-methylbutanoic acids. They have characteristic of odors, being rancid, sweaty, cheesy and sour. The butanoic acid and 2-methylbutanoic acid, acetic acid, propanoic acid and 2-methylpropanoic acid content significantly increased (p<0.05) in the *kung-som* inoculated with *Lc. lactis* KTH0-1S as starter culture compared with spontaneous and CS batches. Among of these compounds, butanoic acid was the major compound regarding to the highest peak area (30.70-44.21%) (Table 19). Butanoic acid was frequently found in various fermented products such as cheeses, fermented sausages and fermented soy bean paste (Yvon and Rijnen, 2001; Shukla *et al*., 2010; Montanari *et al*., 2016). It has a rancid, cheesy, buttery, sour and sweaty odor that contributes to the pleasant aroma (Guarrasi *et al*., 2017). Butanoic acid was formed by pyruvic acid intermediate to be resulted from carbohydrate fermentation via Embden Meyerhof Parnas (EMP) pathway and hexose monophosphate pathway (Shukla *et al*., 2010). It has been reported that *Lactobacillus plantarum* was able to convert lipids into butanoic acid through the activity of intracellular enzymes (Azarnia *et al*., 2006).

In addition, it was noted that 2-methylbutanoic acid and 2-methylpropanoic acid was significantly higher (p<0.05) in *kung-som* inoculated with *Lc. lactis* KTH0-1S. The high signal of 2-methylbutanoic acid and 2-methylpropanoic acid were formed by the conversion of branched-chain amino acids (BCAA, isoleucine and valine) to α-keto acids (α-keto-2-methylbutanoic acid and α-keto-2-methylpropanoic acid intermediates) by aminotransferases produced by *Lc. lactis* strain and then α-keto acids were degraded to aroma compounds (2-methylbutanoic acid and 2-methylpropanoic acid) (Yvon *et al*., 2000; Bourdat-Deschamps *et al*., 2004). This result is strongly in agreement with the study of Yvon *et al*. (2000) who found that the branched-chain aminotransferase (BcaT) activities in *Lc. lactis* subsp. *cremoris* NCDO

763 strain mainly involve BCAA (isoleucine, valine, leucine) and methionine degradation and especially conversion of BCAA to α-keto acids which are also direct precursors of aroma compounds. Several researches revealed that the aroma compounds in fermented foods were generated by enzymatic reactions in microbial cells and amino acids conversion have been reported as a major process for flavors formation (Yvon and Rijnen, 2001; Gutsche *et al*., 2012).

Many publications reported that amino acid conversion to aroma compounds proceeds by 2 different pathways. The first one is initiated by elimination reactions catalyzed by amino acid lyases which cleave the side chain of amino acids. This pathway has been observed for aromatic amino acids and methionine and leads by a single step to phenol, indole and methanethiol, respectively. The second pathway goes through *α*-keto acid intermediates, it is mainly initiated by a transamination reaction catalyzed by amino acid aminotransferases and has been observed for aromatic amino acids, branched-chain amino acids and methionine. The resulting *α*-keto acids are then degraded to aldehydes, alcohols, carboxylic acids, hydroxy acids or methanethiol for methionine via 1 or 2 additional steps (Yvon and Rijnen, 2001; Bourdat-Deschamps *et al*., 2004) Moreover, volatile organic acids such as acetic acid and propanoic acid were mainly generated by carbohydrate fermentation via glycolysis pathway (Shukla *et al*., 2010).

Esters are formed by the esterification of alcohols and carboxylic acids either by microbial esterase activity or by chemical reactions (Majcher *et al*., 2011). *Kung-som* inoculated with *S. carnosus* K2122 as starter culture (CS batch) had higher signal of butyl butanoate (24.97% of relative area), ethyl butanoate (18.26%), 3-methylbutyl butanoate (6.32%) and propyl butanoate (1.84%) than LCS, LS and control batches. On the other hand, inoculated and spontaneous *kung-som* (LCS, CS and C batches) had a higher ethyl 2-methylpropanoate and ethyl phenylacetate than *kung-som* in LS batch. The high signal of ester compounds in *kung-som* produced by *S. carnosus* K21S22 as starter culture was result from the Staphylococcal esterase activities which hydrolyzed esters with different acids or esterified alcohols with acids (Talon *et al*., 1998; Gao *et al*., 2018). The obtained result was in accordance with study of Jeong *et al*. (2016) who found that the addition of *S. succinus* 14BME20 as starter culture was significantly high with level of 3-methylbutyl acetate comparison with other batches.

Fonseca *et al*. (2013) also reported that Spanish fermented sausage inoculated with *S. epidermidis* SA49 or *S. saprophyticus* B12 as starter culture gave a significantly higher of volatile esters content than spontaneous one. However, in the present study we found that *kung-som* inoculated with *S. carnosus* K21S22 as starter culture (CS and LCS batches) showed a significantly lower of alcohol signal ($p<0.05$) than *kung-som* inoculated with *Lc. lactis* KTH0-1S (LS batch) and spontaneous sample.

The low level of alcohol in CS and LCS may be resulted from the alcohols conversion to volatile ester compounds by esterase activity produced by *S. carnosus* K21S22. This is in accordance with study of Jeong *et al*. (2016) reported that the volatile alcohols content of sample inoculated with *S. succinus* 14BME20 were significantly lower than control treatment. Alcohols including ethanol, butan-1-ol, 1-pentenol, 1-octen-3-ol, (5Z)-oct-1,5-dien-3-ol, butane-2, 3-diol, (2R, 3R)-butane-2, 3-diol have been reported to be generated from the reduction of aldehydes by lipid oxidation phenomenon (Tabanelli *et al*., 2012; Jeong *et al*., 2016). These alcohols gave the pleasant flavors with sweet, fruity or buttery, mushroom and green-like flavors (Latorre-Moratalla *et al*., 2011; Zhao *et al*., 2011; Bosse *et al*., 2017). Furthermore, phenols, pyrazines, butan-1-amine, 2-piperidinone and indole were detected in less amount for all *kung-som* samples, those contributed to phenolic, nutty, ammonia-like and earthy odor, respectively (Good Scent Company Information System, 2018). To the best of our knowledge, the present study is the first report to identify the abundant flavor in *kung-som*. The addition of *Lc. lactis* KTH0-1S or/and *S. carnosus* K21S22 as starter culture in *kung-som* fermentation significantly affected to the volatile acids, esters and alcohol formation in finished *kung-som* products.

Table 19 Effect of spontaneous and inoculation of autochthonous on the production of active volatile compounds in *kung-som* product.

No.	Compounds	Chemical group	Relative peak area[A]				Odor description
			C*	LS	CS	LCS	
1	ethyl acetate	Ester	0.11^{c}	0.38^{a}	0.09^{d}	0.13^{b}	Fruity, sweet, green
2	ethanol	Alcohol	1.86^{b}	3.18^{a}	0.52^{d}	0.87^{c}	Alcoholic
3	methyl butanoate	Ester	2.33^{a}	0.38^{c}	0.39^{c}	0.66^{b}	Fruity, fermented
4	ethyl butanoate	Ester	16.42^{b}	4.72^{c}	18.26^{a}	17.00^{a}	Fruity, sweet, apple
5	propyl butanoate	Ester	1.50^{b}	0.02^{d}	1.84^{a}	1.21^{c}	Fruity, sweet, pineapple
6	butan-1-ol	Alcohol	0.49^{a}	nd^{c}	0.32^{b}	nd^{c}	Sweet, banana
7	butyl butanoate	Ester	15.76^{b}	1.76^{d}	24.97^{a}	4.23^{c}	Sweet, fruity, fatty
8	3-methylbutyl butanoate	Ester	2.94^{c}	0.84^{d}	6.32^{a}	5.68^{b}	Fruity, sweet, green,
9	2-ethyl-3,5-dimethylpyrazine	Pyrazine	0.53^{a}	0.35^{b}	0.37^{b}	0.33^{b}	Sweet, nutty, coffee
10	1-octen-3-ol	Alcohol	0.09^{a}	nd^{b}	0.10^{a}	0.09^{a}	Mushroom, earthy, green
11	acetic acid	Carboxylic acid	0.51^{c}	2.31^{a}	0.15^{d}	0.79^{b}	Pungent, sour
12	(5Z)-oct-1,5-dien-3-ol	Alcohol	0.11^{a}	0.13^{a}	0.06^{b}	0.04^{b}	Green, marine, mushroom
13	butane-2,3-diol	Alcohol	0.88^{c}	1.97^{a}	0.59^{d}	1.45^{b}	Fruity, creamy, buttery
14	propanoic acid	Carboxylic acid	0.71^{b}	1.36^{a}	0.44^{d}	0.59^{c}	Acidic, dairy, fruity
15	2-methylpropanoic acid	Carboxylic acid	0.41^{c}	1.84^{a}	0.31^{d}	0.62^{b}	Cheesy, creamy, sour
16	(2R,3R)-butane-2,3-diol	Alcohol	0.67^{b}	0.81^{a}	0.45^{d}	0.52^{c}	Fruity, creamy, buttery
17	butanoic acid	Carboxylic acid	41.02^{c}	44.21^{b}	30.70^{d}	46.23^{a}	Acidic, sour, cheesy
18	2-methylbutanoic acid	Carboxylic acid	7.43^{c}	25.22^{a}	5.46^{d}	12.50^{b}	Fruity, buttery, cheesy
19	3-methylbutanoic acid	Carboxylic acid	1.24^{a}	1.23^{a}	1.26^{a}	1.39^{a}	Cheesy, creamy, sweet
20	ethyl 2-methylpropanoate	Ester	0.76^{a}	0.25^{c}	0.64^{b}	0.70^{a}	Pungent, fruity, sweet
21	pentanoic acid	Carboxylic acid	0.10^{c}	0.09^{c}	0.32^{b}	0.45^{a}	Acid cheesy
22	ethyl phenylacetate	Ester	0.36^{a}	0.11^{b}	0.15^{b}	0.37^{a}	Sweet, rosy, honey
23	Butan-1-amine	Amine	0.13^{a}	0.04^{b}	0.14^{a}	0.15^{a}	Fishy, ammonia-like,
24	2-phenylethanol	Phenol	0.40^{b}	0.28^{c}	0.59^{a}	0.52^{a}	Flora sweet
25	phenol	Phenol	0.97^{a}	1.03^{a}	0.88^{b}	0.81^{b}	Phenolic
26	4-methylphenol	Phenol	0.17^{a}	0.15^{ab}	0.19^{a}	0.11^{b}	phenolic
27	2-piperidinone	Lactam	0.19^{b}	nd^{d}	1.33^{a}	0.09^{c}	Ammonia-like
28	indole	Heterocycle	0.05^{b}	0.02^{c}	0.15^{a}	0.03^{bc}	Earthy, phenolic

[A]Values presented as the average of three replicates are the relative peak areas.

[a-d]Different superscript within a row represent significant differences (p<0.05); nd: non-detecable.

*Batch C: natural fermented *kung-som*, Batch LS: *kung-som* inoculated with only nisin Z-producing *Lc. lactis* KTH0-1S as a starter culture, Batch CS: *kung-som* inoculated with only *S. carnosus* K21S22 as a starter culture and Batch LCS: *kung-som* inoculated with co-culture of *Lc. lactis* KTH0-1S and *S. carnosus* K21S22.

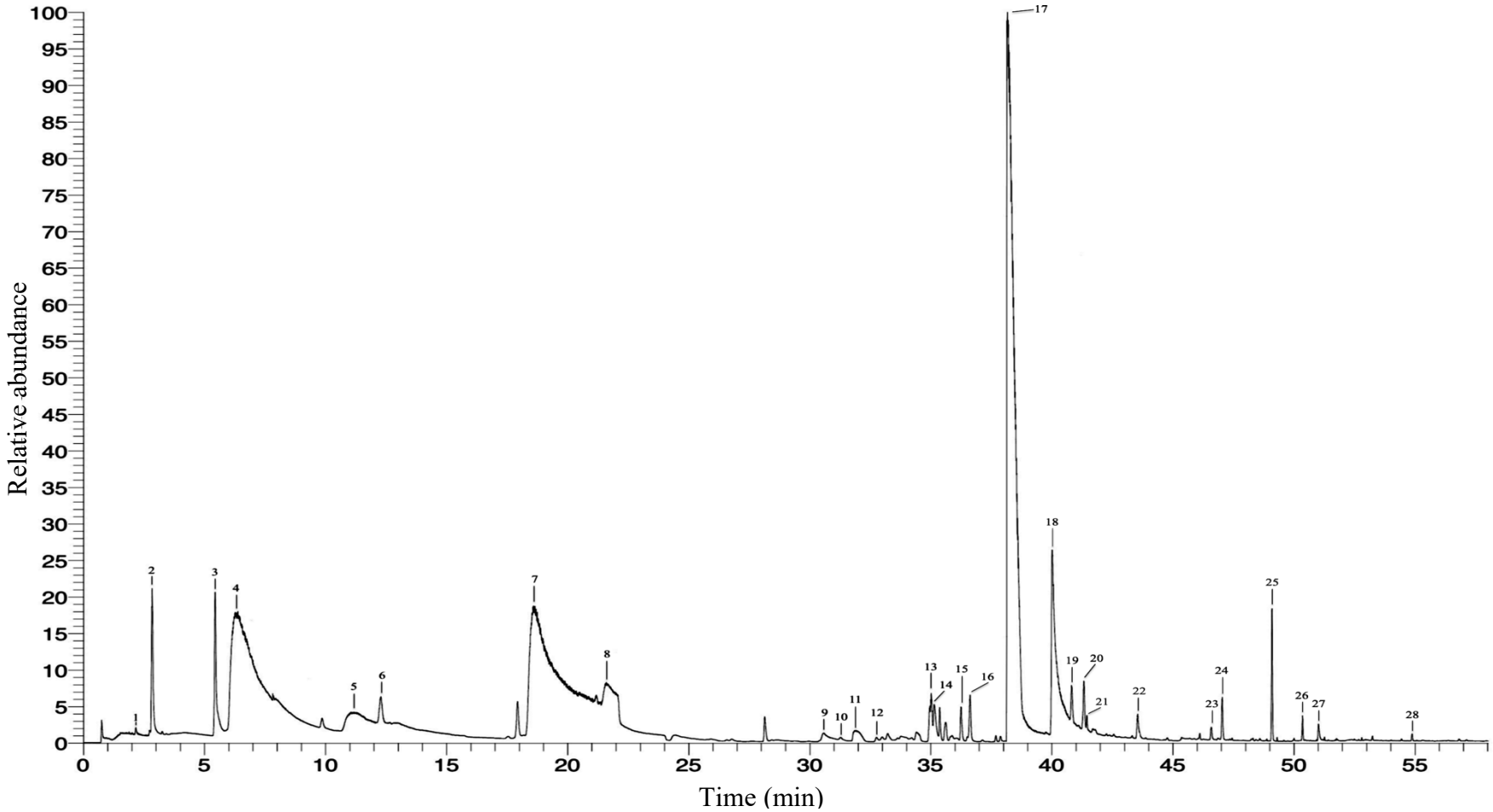

Figure 41 GC chromatogram of volatile compounds obtained from the spontaneous *kung-som*. The number on each peak of GC chromatogram is referred to the type of volatile compound are shown in Table 19.

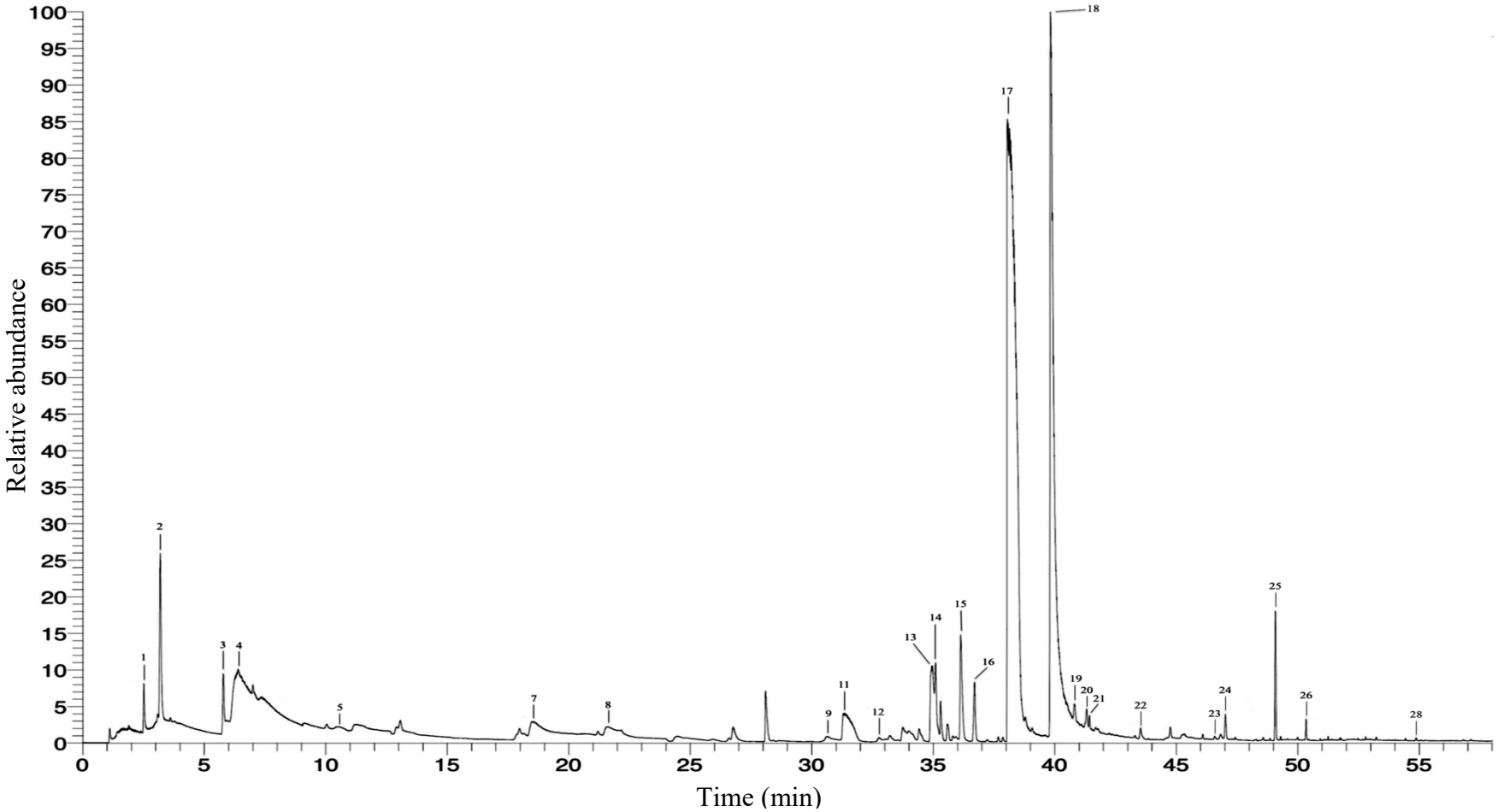

Figure 42 GC chromatogram of volatile compounds obtained from *kung-som* inoculated with *Lc. lactis* KTH0-1S as starter culture. The number on each peak of GC chromatogram is referred to the type of volatile compound are shown in Table 19.

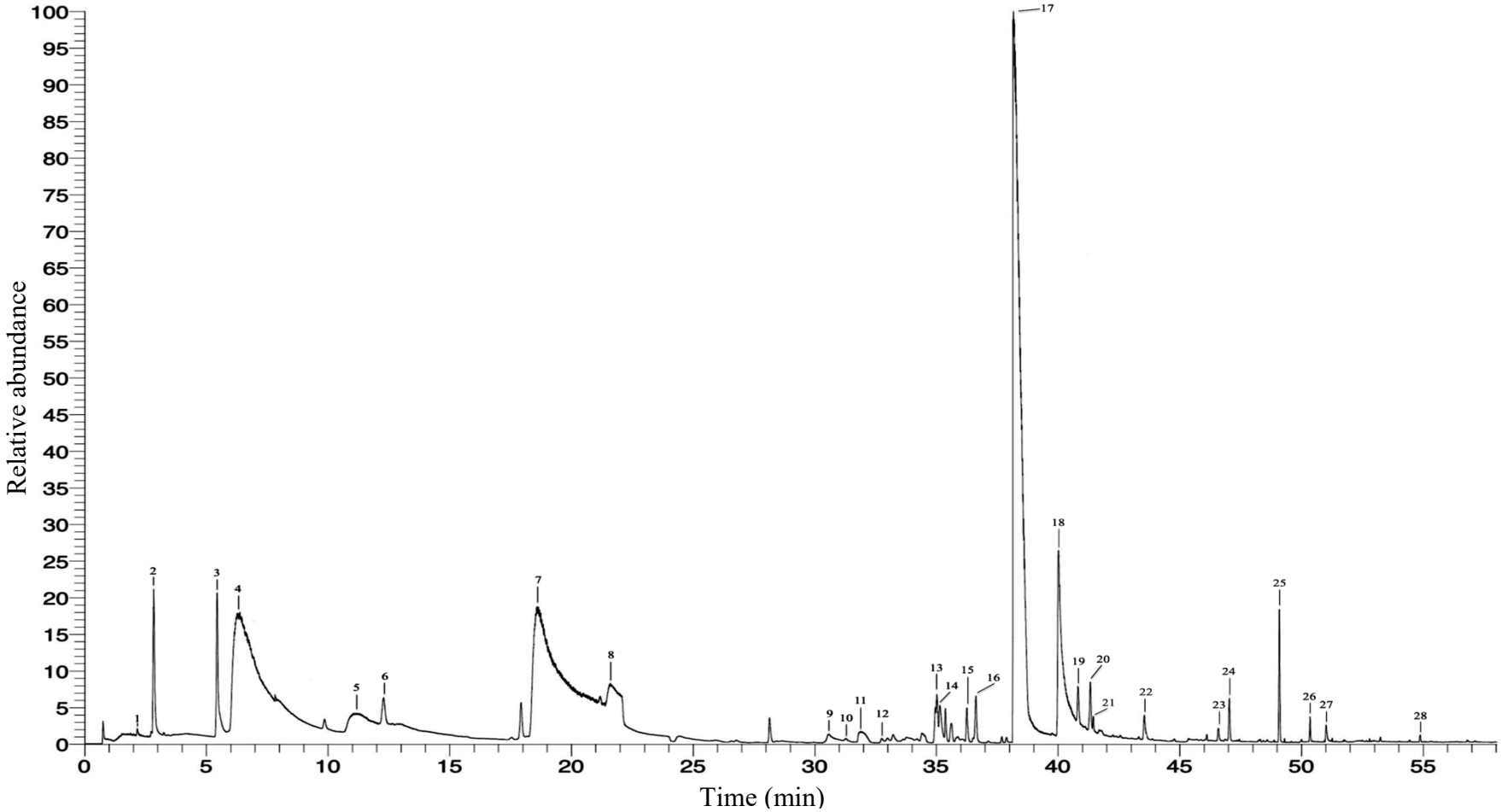

Figure 43 GC chromatogram of volatile compounds obtained from *kung-som* inoculated with *S. carnosus* K21S22 as starter culture. The number on each peak of GC chromatogram is referred to the type of volatile compound are shown in Table 19.

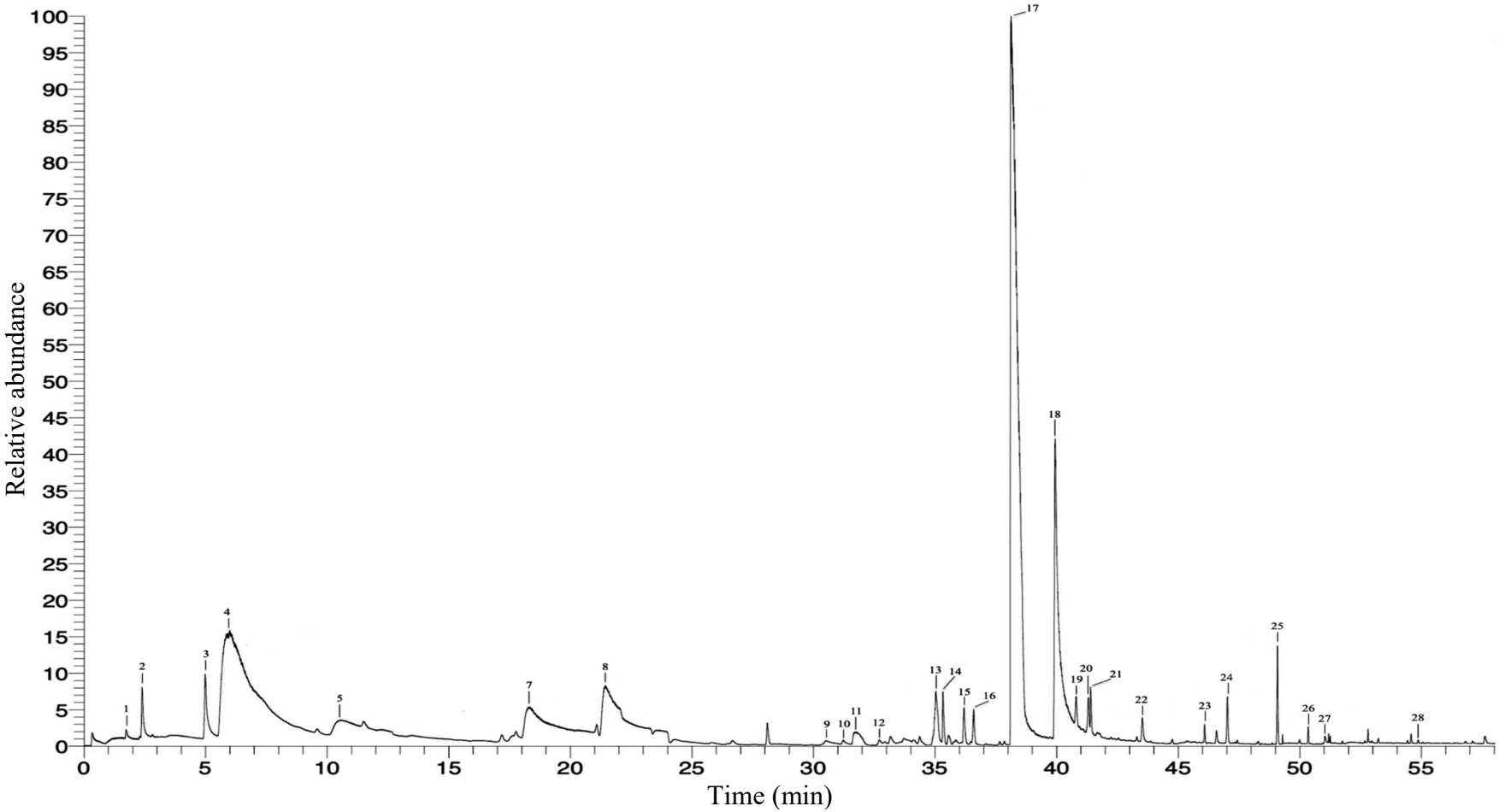

Figure 44 GC chromatogram of volatile compounds obtained from *kung-som* inoculated with *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter culture. The number on each peak of GC chromatogram is referred to the type of volatile compound are shown in Table 19.

5.4.4.3 Sensory evaluation

The sensory characteristics of *kung-som* produced by the addition with and without starter culture were evaluated. The sensory results were a significant difference between *Lc. lactis* KTH0-1S or *S. carnosus* K21S22-inoculated *kung-som*, mixed starter culture-inoculated *kung-som* and spontaneous *kung-som*. *Kung-som* inoculation with only *Lc. lactis* strain (LS batch) had the highest score for all tested characteristics. This result is in agreement with the study of Sanchart *et al.* (2017) who reported that *kung-som* produced by the addition of *L. futsaii* CS3 as starter culture gave the desirable sensory characteristic. However, Hwanhlem *et al.* (2010) found that inoculated *kung-som* with *Lb. plantarum* D6SM3 had a lower sensory score than naturally fermented *kung-som.* Additionally, *kung-som* produced by an inoculation with mixed starter culture had a significant higher score than the C and CS batches ($p<0.05$) for color, taste, flavor, texture as well as overall acceptance. *Kung-som* inoculated with only *S. carnosus* K21S22 (CS batch) showed an adverse effect for all sensory characteristics (Table 20 and Fig. 45). The obtained negative effects (Table 20) were due to the competition effect of high load *S. carnosus* K21S22 against the bacterial flora (Kuda *et al*., 2012; Tabanelli *et al*., 2014) and affect to decrease of LAB (Fig. 40C). This might be affected to the LAB metabolites production including organic acids as well as volatile compounds production which were markedly influence on organoleptic quality including color, flavor, texture as well as overall acceptance of *kung-som* product.

Our results demonstrated that the inoculation with *Lc. lactis* KTH0-1S as starter culture in *kung-som* significantly reduced the pathogenic and spoilage bacteria as well as enhanced the volatile compound including acids and alcohols in the finished *kung-som* when compared to the others batch. Additionally, the *kung-som* produced by inoculation with *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter showed the higher scores than CS and control batches. In contrast, *kung-som* inoculated with only *S. carnosus* K21S22 as starter culture resulted in changes of ratio of LAB and non-LAB as microflora to be affected the quality of *kung-som*. Therefore, *Lc. lactis* KTH0-1S had potential to be used as autochthonous starter culture for improving safety and quality of *kung-som* product.

Table 20 Sensory evaluation liking scores of spontaneous and inoculated *kung-som*.

Sensory Characteristics	**Liking scores**			
	Spontaneous (C batch)	Inoculated with *Lc. lactis* (LS batch)	Inoculated with *S. carnosus* (CS batch)	Inoculated with both strains (LCS batch)
Appearance	5.33±1.71^{c}	8.03±0.93^{a**}	4.07±1.64^{d}	7.27±1.08^{b}
Color	5.47±1.66^{c}	7.83±1.12^{a}	3.67±1.49^{d}	7.07±1.05^{b}
Taste	4.67±2.14^{b}	6.60±1.65^{a}	3.87±1.85^{c}	6.10±1.71^{a}
Flavor	5.10±1.84^{b}	6.57±1.28^{a}	4.43±1.61^{c}	6.00±1.55^{a}
Texture	4.70±2.17^{b}	6.77±1.50^{a}	3.83±1.84^{c}	6.27±1.57^{a}
Overall acceptance	5.07±1.78^{c}	7.40±1.07^{a}	4.10±1.71^{d}	6.60±1.45^{b}

Values are given as mean ± standard deviation (n=30).
*p<0.05 is considered a significant difference (ANOVA).
**Different superscripts in the same row indicate significant differences (p<0.05).

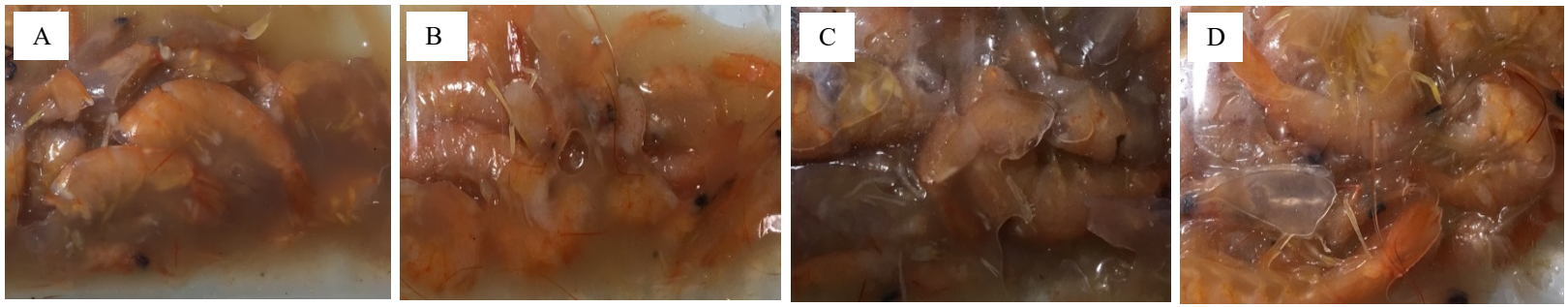

Figure 45 Appearance of *kung-som* produced by spontaneous (C batch) (A), inoculated with *Lc. lactis* KTH0-1S as a starter culture (LS batch) (B), inoculated with *S. carnosus* K21S22 as a starter culture (CS batch) (C) and inoculated with *Lc. lactis* KTH0-1S and *S. carnosus* K21S22 as starter culture (LCS batch) (D).

5.5 Conclusion

Our finding demonstrated that *S. carnosus* K21S22 was selected due to the absence of biofilm formation, hemolytic activity, and further virulence or toxigenic properties. This strain could be tolerated at high salt concentration and produced protease and lipase enzymes. The production of *kung-som* by inoculation with an autochthonous starter culture (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) was significantly affecting on microbial counts, acidity, volatile compounds and organoleptic properties in samples. The inoculation of *Lc. lactis* KTH0-1S as starter culture in *kung-som* (LS and LCS batches) could be reduced the fermentation time and improved microbiological safety. Furthermore, *kung-som* produced by addition of only *Lc. lactis* KTH0-1S and mixed starter culture (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) was significantly positive effect on sensory properties when compared with control and CS batch. However, the addition of only *S. carnosus* K21S22 as starter culture gave an adverse effect on *kung-som* quality. Accordingly, *kung-som* produced by the inoculation of *Lc. lactis* KTH0-1S gave a good result in more safety, more organoleptic properties and shorten time period of fermentation than other *kung-som* treatments.

CHAPTER 6

SUMMARY AND SUGGESTION

6.1 Summary

To the best of our knowledge, a traditional Thai fermented shrimp (*kung-som*) distributed in local markets was safe for human consumption. The profile of bacteria flora in *kung-som* was investigated by using DGGE technique that is mainly various LAB and CNS. Nisin Z-producing *Lactococcus lactis* KTH0-1S was isolated from *kung-som* that exhibited inhibitory activity in wide range of pH, high temperature and sterile condition. This strain is considered as good candidate to be used as a starter culture or protective culture in fermented food products due to its desirable technological and safety characteristics. Additionally, nisin Z-producing *Lc. lactis* KTH0-1S significantly decreased the growth of tyraminogenic bacteria and tyramine production *in vitro* condition. The addition of *Lc. lactis* KTH0-1S as a starter culture could inhibit the indigenous microbiota and increase the effectiveness of the tyramine reduction during *kung-som* fermentation. Moreover, *Staphylococcus carnosus* K21S22 isolated from *kung-som* as CNS strain was safe and showing positive technological properties to be selected as starter culture in *kung-som* production.

Kung-som produced by an inoculation with the autochthonous starter culture (*Lc. lactis* KTH0-1S and *S. carnosus* K21S22) was significantly affecting on microbiological safety and organoleptic properties. The addition of *Lc. lactis* KTH0-1S as starter culture in *kung-som* (LS and LCS batches) could be decreased the periods time of fermentation and enhanced safety. Furthermore, *kung-som* produced by the inoculation of only *Lc. lactis* KTH0-1S and mixed starter culture (LS and LCS batches) was significantly positive effect on sensory properties when compared with spontaneous and CS batch. However, the addition of only *S. carnosus* K21S22 as a starter culture gave an adverse effect on *kung-som* quality. Therefore, *kung-som* production by the addition of *Lc. lactis* KTH0-1S as starter cultures provide a good result in an enhancement of safety and improvement of *kung-som* quality.

6.2 Suggestion

1. Our results demonstrated that nisin Z-producing *Lc. lactis* KTH0-1S successfully suppressed undesirable bacteria and also reduced the tyramine accumulation in *kung-som*. However, the preparation of *Lc. lactis* KTH0-1S in fresh starter culture is difficult to handle in commercial scale. It should be further prepared the starter culture in dry form to facilitate its commercialization and use.

2. Although *kung-som* produced by addition of only *S. carnosus* K21S22 showed negative effect on sensory quality of *kung-som*, this strain had the desirable technological characteristics and safety properties indicating it has potential for further application in other Thai traditional fermented products.

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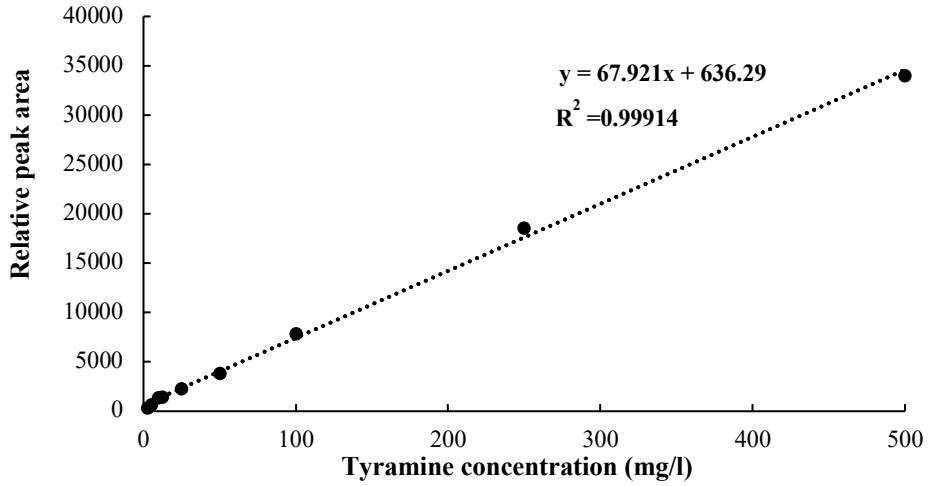
APPENDIX

APPENDIX A

Standard curve of tyramine standard



Retention time (min)	Level	Amount (mg/l)	Area
10.530	1	2.5	336.087
	2	5	696.616
	3	10	1389.180
	4	12.5	1444.744
	5	25	2319.928
	6	50	3875.800
	7	100	7910.500
	8	250	18577.000
	9	500	34041.000

APPENDIX B

BLAST results of nucleotide of 16S rDNA from *Lactococcus lactis* KTH0-1S

Lactococcus lactis NBRC 100933 16S ribosomal RNA gene, partial sequence

Score	Expect	Identities	Gaps	Strand
2560 bits(1386)	0.0	1424/1441(99%)	8/1441(0%)	Plus/Plus

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Query  2     GGGGGGGTGCCTA-TA-ATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCGACTGGAT  59
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Query  60    GAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTG  119
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Query  120   GAAACGAATGCTAATACCGCATAACAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCA  179
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BLAST results of nucleotide of 16S rDNA from *Enterococcus faecalis* 9Y

Enterococcus faecalis ATCC 19433 16S ribosomal RNA gene, partial sequence

Score	Expect	Identities	Gaps	Strand
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Sbjct  455   GTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA  514
Query  481   GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCA  574
Query  541   GGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575   GGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC  634
Query  601   TGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635   TGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG  694
Query  661   ATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  695   ATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCT  754
Query  721   CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  755   CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG  814
Query  781   TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  815   TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGC  874
Query  841   CTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  875   CTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG  934
Query  901   TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  935   TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTT  994
Query  961   GACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGT  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  995   GACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGT  1054
Query  1021  CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1055  CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTT  1114
Query  1081  AGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGT  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1115  AGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGT  1174
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Query  1141  GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGG  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1175  GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGG  1234
Query  1201  AAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTC  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1235  AAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTC  1294
Query  1261  GGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1295  GGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCA  1354
Query  1321  CGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTG  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1355  CGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTG  1414
Query  1381  TAACACCCGAAGTCGGTGAGGT  1402
             ||||||||||||||||||||||
Sbjct  1415  TAACACCCGAAGTCGGTGAGGT  1436
```

BLAST results of nucleotide of 16S rDNA from *Staphylococcus carnosus* K21S22

Staphylococcus carnosus CIP103274 16S ribosomal RNA gene, partial sequence

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Score             Expect       Identities             Gap              Stand
2518 bits (2792)  0.0          1405/1411(99%)         0/1411(0%)       Plus/Plus
Query  4     CGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGG  63
             |||||||| |||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  60    CGAACAGATGAGGAGCTTGCTCCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGG  119
Query  64    TAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAATATGC  123
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   TAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAATATGC  179
Query  124   GGAACCGCATGGTTCCGCAATGAAAGACGGTTTTGCTGTCACTTATAGATGGACCCGCGC  183
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   AGAACCGCATGGTTCTGCAATGAAAGACGGTTTTGCTGTCACTTATAGATGGACCCGCGC  239
Query. 184   CGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAG  243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct. 240   CGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAG  299
Query. 244   AGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTA  303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300   AGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTA  359
Query  304   GGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTC  363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   GGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTC  419
Query  364   TTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAGTGCGTAGGTAACTATGCGCACCT  423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct. 420   TTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAGTGCGTAGGTAACTATGCGCACCT  479
Query. 424   TGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480   TGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA  539
Query  484   GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTC  543
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540   GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTC  599
Query  544   TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGC  603
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600   TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGC  659
Query  604   AGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACAC  663
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   AGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACAC  719
Query  664   CAGTGGCGAAGGCGACTTTCTGGTCTGCAACTGACGCTGATGTGCGAAAGCGTGGGGATC  723
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720   CAGTGGCGAAGGCGACTTTCTGGTCTGCAACTGACGCTGATGTGCGAAAGCGTGGGGATC  779
Query  724   AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGG  783
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  780   AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGG  839
Query  784   GTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCG  843
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  840   GTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCG  899
Query  844   CAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA  903
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  900   CAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA  959
Query  904   ATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATA  963
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  960   ATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATA  1019
Query  964   GAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC  1023
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1020  GAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC  1079
Query  1024  GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAACTTAGTTGCCAGCATTA  1083
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1080  GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTA  1139
Query  1084  AGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA  1143
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1140  AGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA  1199
Query  1144  ATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAGTACAAAGGGCA  1203
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1200  ATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAGTACAAAGGGCA  1259
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Query  1204  GCGAAACCGCGAGGTCAAGCAAATCCCATAAAGCTGTTCTCAGTTCGGATTGTAGTCTGC  1263
             ||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  1260  GCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGC  1319
Query  1264  AACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA  1323
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1320  AACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA  1379
Query  1324  CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTCGTAACACCCGAAGCC  1383
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1380  CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTCGTAACACCCGAAGCC  1439
Query  1384  GGTGGAGTAACCTTTTAGGAGCTAGCCGTCG  1414
             ||||||||||||||||||||||||||||||
Sbjct  1440  GGTGGAGTAACCTTTTAGGAGCTAGCCGTCG  1470
```

The nucleotide sequence of *ldc* gene was amplified with specific primers from *S. carnosus* K21S22. Its sequence was 99% similarity of 16S rDNA gene of *Staphylococcus* sp.

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Query    1  CTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAA  60
           |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    59 CTCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAA  118
Query    61 TAACTCCGGGAAACCGGGGCTAATGCCGGATAATATGCGGAACCGCATGGTTCCGCAATG  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   119 TAACTCCGGGAAACCGGGGCTAATGCCGGATAATATGCGGAACCGCATGGTTCCGCAATG  178
Query   121 AAAGACGGTTTTGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGT  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   179 AAAGACGGTTTTGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGT  238
Query   181 AACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAA  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   239 AACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAA  298
Query   241 CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGA  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   299 CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGA  358
Query   301 AAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTA  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   359 AAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTA  418
Query   361 TTAGGGAAGAACAAGTGCGTAGGTAACTATGCGCACCTTGACGGTACCTAATCAGAAAGC  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   419 TTAGGGAAGAACAAGTGCGTAGGTAACTATGCGCACCTTGACGGTACCTAATCAGAAAGC  478
Query   421 CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAAT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   479 CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAAT  538
Query   481 TATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCA  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   539 TATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCA  598
Query   541 ACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCA  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   599 ACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCA  658
Query   601 TGTGTAGCGGTGAAATGCGCAGGGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTG  660
           |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct   659 TGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTG  718
Query   661 GTCTGCAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGT  720
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   719 GTCTGCAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGT  778
Query   721 AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA  780
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   779 AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA  838
Query   781 GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT  840
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   839 GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT  898
Query   841 TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC  900
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   899 TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC  958
Query   901 TTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGGGGGACA  960
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   959 TTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGGGGGACA  1018
Query   961 AAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC  1020
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019 AAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC  1078
Query  1021 GCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAAGTTGGGCACTCTAAGTTGACT  1080
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1079 GCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAAGTTGGGCACTCTAAGTTGACT  1138
Query  1081 GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTT  1140
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1139 GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTT  1198
Query  1141 GGGCTACACACGTGCTACAATGGACAGTACAAAGGGCAGCGAAACCGCGAGGTCAAGCAA  1200
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1199 GGGCTACACACGTGCTACAATGGACAGTACAAAGGGCAGCGAAACCGCGAGGTCAAGCAA  1258
Query  1201 ACCCCATAAAGCTGTTCTCAGTTCGGATTGTAGTCT  1236
           |||||||||||||||||||||||||||||||||||
Sbjct  1259 ATCCCATAAAGCTGTTCTCAGTTCGGATTGTAGTCT  1294
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VITAE

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Educational Attainment

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Master of Science (Biotechnology)	Prince of Songkla University	2010

Scholarship Awards during Enrolment

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- Graduate School, Prince of Songkla University.
- The French Bio-Asie project "LAB-Utilization of lactic acid bacteria in foods and health" from French Foreign Affair Ministry.

List of Publications and Presentations

Publications

1. **Saelao, S.**, Kaewsuwan, S., Benjakul, S. and Maneerat, S. 2016. Safety evaluation and bacterial community of *kung-som* using PCR-DGGE technique. Songklanakarin J. Sci Technol. 38: 413-419.
2. **Saelao, S.**, Maneerat, S., Kaewsuwan, S., Rabesona, H., Choiset, Y., Haertlé, T. and Chobert, J. M. 2017. Inhibition of *Staphylococcus aureus* in vitro by bacteriocinogenic *Lactococcus lactis* KTH0-1S isolated from Thai fermented shrimp (*Kung-som*) and safety evaluation. Arch. Microbiol. 199: 551-562.
3. **Saelao, S.**, Maneerat, S., Thongruck, K., Watthanasakphuban, N., Wiriyagulopas, S., Chobert, J. M. and Haertlé, T. 2018. Reduction of tyramine accumulation in Thai fermented shrimp (*kung-som*) by nisin Z-producing *Lactococcus lactis* KTH0-1S as starter culture. Food Control 90: 249-258.

4. **Saelao, S.**, Maneerat, S., Wiriyagulopas, S., Haertlé, T. and Chobert, J. M. 2018. Selection of save coagulase-negative staphylococci technologically useful as starter culture for production of Thai fermented shrimp (*kung-som*). Int. J. Food Microbiol. (Submitted)

Presentations

1. **Saelao, S.**, Kaewsuwan, S. and Maneerat, S. 2012. Microbiological quality and bacterial composition of *Kung-Som*-a fermented shrimp. The 1st International Conference on Microbial Taxonomy, Basic and Applied Microbiology. Kosa Hotel, Khon Kaen, Thailand. October 4-6th, 2012. pp. 67.
2. **Saelao, S.**, Kaewsuwan, S., Chobert, J. M. and Maneerat, S. 2015. Characterization of nisin Z producing *Lactococcus lactis* KTH0-1S and safety assessment for used as starter culture in Thai fermented shrimp (*kung-som*). The 8th Asian Conference for Lactic Acid Bacteria (ACLAB). The Emerald Hotel, Bangkok, Thailand. July 8-10th, 2015. pp. 111.