

รายงานวิจัยฉบับสมบูรณ์

โครงการการเปลี่ยนแปลงทางจุลินทรีย์และทางเคมีของหมึกกล้วย ที่ใช้วิธีถนอมอาหารแบบร่วมในระหว่างการเก็บรักษาแบบแช่เย็น

โดย ผู้ช่วยศาสตราจารย์ ดร.ปุณณาณี สัมภวะผล

กันยายน 2563

สัญญาเลขที่ MRG5580103

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ผู้ช่วยศาสตราจารย์ ดร.ปุณณาณี สัมภวะผล
คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

แบบสรุปปิดโครงการวิจัย *(จัดทำแยกต่างหากจากรายงานฉบับสมบูรณ์)*

สัญญาเลขที่ MRG5580103 ชื่อโครงการ การเปลี่ยนแปลงทางจุลินทรีย์และทางเคมีของหมึกกล้วยที่ใช้วิธีถนอมอาหารแบบร่วมในระหว่างการเก็บรักษาแบบแช่เย็น หัวหน้าโครงการ ผู้ช่วยศาสตราจารย์ ดร.ปุณณาณี สัมภวะผล หน่วยงาน คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ โทรศัพท์ 074286366 โทรสาร 074558866 อีเมล์ punnanee.s@psu.ac.th

สถานะผลงาน ☑ **ปกปิด** ☐ **ไม่ปกปิด**

ความสำคัญ / ความเป็นมา

The exploitation of cephalopods has increased in recent years as a consequence of the decline stocks of commonly exploited fish species. The world demand for cephalopods increased greater than that related for marine fishes, indicating a specific demand for these marine animals. Squids are the main marine animals in the world cephalopod fishery production, mainly due to their excellent sensory properties (Sikorski and Kolodziejska, 1986). *Loligo formosana* (*L. chinensis*) is the economically important squid in the Southeast Asia. This squid is caught along Western Pacific Southern Japan to Northern and Northeastern Australia. In Thailand, *L. formosana* has been caught along the Gulf of Thailand and Andaman sea. It is very important species of Thailand and its common name is "Splendid squid" (Department of Fisheries, 2006). Among 10 kinds of squids caught in Thailand, *L. formosana* is caught for about 12.65% of the total squid catch (Department of Fisheries, 2006).

During handling or storage, cephalopods undergo changes in texture, flavor and color. Quality changes of squids are generally dependent on storage condition (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Lapa-Guimaraes *et al*., 2002; Lapa-Guimaraes *et al*., 2005). The pH, total volatile base (TVB) and polyamine of squid increased with increasing storage temperatures (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Sungsri-in *et al*., 2011). Early studies of seafood microbiology indicated that only part of the spoilage microflora participated in the spoilage process (Huss *et al*., 1974; Castell and Anderson, 1948). Nevertheless, the recent establishment of the specific spoilage organism (SSO) concept (Dalgaard, 2000) has contributed significantly to our understanding of seafood spoilage. The SSOs are typically present in low numbers and constitute only a very small fraction of the microflora on newly processed seafood. Different SSOs are found in different seafoods (Dalgaard, 2000). Identification of an SSO relies on comparison of the sensory and chemical characteristics of spoiled products with those of the spoilage microflora. The qualitative ability to produce off-odors (spoilage potential) and the quantitative ability to produce spoilage metabolites (spoilage activity) are essential in the identification of an SSO (Dalgaard, 2000). Quantitative comparison of the production of TMA, biogenic amines or volatile amines in products and that mediated by bacterial isolates grown in model substrate are useful for the identification of SSOs (Jorgensen *et al*., 2000; Dalgaard, 1995; Koutsoumanis and Nychas, 2000). Comparison of the chemical profiles of spoiled seafoods and of the metabolites produced by potential spoilage organisms has only been used to a limited extent in identification of SSOs. The spoilage domain has been defined as the range of conditions (pH, temperature, water activity and atmosphere) under which an SSO can grow and produce spoilage metabolites (Dalgaard, 2000). Therefore, the identification of SSOs and their spoilage domains could facilitate the development of methods to determine, predict and extend the shelf life of squid. However, there is very little information on the role of specific spoilage microorganisms in the quality loss of squid during

postmortem handling and storage. Therefore, this study aim to identify the major spoilage microorganisms in squid during refrigerated storage.

วัตถุประสงค์ของโครงการ

1. To monitor the microbiological and chemical changes of squid during refrigerated storage
2. To identify the specific spoilage organisms of squid during refrigerated storage
3. To study the impact of some antimicrobial agents on the microbiological and chemical changes of squid during refrigerated storage
4. To study the effect of packaging atmospheres on the microbiological and chemical changes of squid during refrigerated storage
5. To investigate microbiological and chemical changes of squid preserved by combined methods during refrigerated storage

ผลการวิจัย *(สั้น ๆ ที่บ่งชี้ประเด็นข้อค้นพบ กระบวนการ ผลผลิต และการเรียนรู้)*

Squids are the main marine animals in the world cephalopod fishery production. During handling or storage, squids undergo changes in texture, flavor as well as color, leading to less acceptability. Quality changes of squids are generally dependent on storage conditions including storage temperature and time. Generally, the rapid growth of microorganisms is associated with the spoilage of squid. However, there is very little information on the role of specific spoilage microorganisms in the quality loss of squid during postmortem handling and storage. Therefore, this study aims to identify the major spoilage microorganisms in squid during refrigerated storage. And further, elucidate the combination method to prolong the shelf-life of squid keep at refrigerated temperature. All squid samples (1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning) were stored in a refrigerator (4°C) for 14 days. During storage, it was found that microbial count, TVB, TMA contents, pH and color of squid undergo changes and spoiled within 2 days of storage. *Aeromonas aeroginosa* was found to be a specific spoilage organism of squid due to it accelerates the quality changes of squid compared to other identified microorganisms. Moreover, squid treated with citric acid could retard the shelf-life of squid from 2 to 4 days while MAP could extend the shelf-life from 2 to 6 days. A combination of citric acid treated with MAP could extend the shelf-life of squid (whole squids, squids with evisceration and squids with evisceration and skinning) from 2 to 8 days of storage. Further identified the associate of *Aeromonas aeroginosa* and other microorganisms caused spoilage of squid could be fruitfully to seafood industries.

คำสืบค้น (Keywords)

Squid; Refrigerated storage; Microbiological change; Chemical change

การนำผลงานวิจัยไปใช้ประโยชน์ *(ดูคำจำกัดความ และตัวอย่างด้านหลังแบบฟอร์ม)*

☐ **ด้านนโยบาย** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)*.....

มีการนำไปใช้อย่างไร

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☐ **ด้านสาธารณะ** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

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☐ **ด้านชุมชนและพื้นที่ โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

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☐ **ด้านพาณิชย์ โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

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☑ **ด้านวิชาการ โดยใคร**

- ภาควิชาเทคโนโลยีอาหาร คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์
- สถาบันการศึกษาต่างๆ

มีการนำไปใช้อย่างไร *(กรุณาให้ข้อมูลเจาะจง)*

ผลที่ได้จากการศึกษาของโครงการ "การเปลี่ยนแปลงทางจุลินทรีย์และทางเคมีของหมึกกล้วยที่ใช้วิธีถนอมอาหารแบบร่วมในระหว่างการเก็บรักษาแบบแช่เย็น" ทำให้เกิดเอกสารประกอบการสอน หัวข้อ การเสื่อมเสียของอาหารโดยจุลินทรีย์ ซึ่งมีการเรียนการสอนในรายวิชา 850-333 จุลชีววิทยาทางอาหาร 2 สำหรับนักศึกษาสาขาวิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์

☐ **ยังไม่มีการนำไปใช้** (โปรดกรอกในกรอบถัดไป)

(กรณีที่ยังไม่มีการใช้ประโยชน์) **ผลงานวิจัยมีศักยภาพในการนำไปใช้ประโยชน์**

☐ **ด้านนโยบาย** ☐ **ด้านสาธารณะ** ☐ **ด้านชุมชนและพื้นที่** ☐ **ด้านพาณิชย์** ☐ **ด้านวิชาการ**

ข้อเสนอแนะเพื่อให้ผลงานถูกนำไปใช้ประโยชน์

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การเผยแพร่/ประชาสัมพันธ์ *(กรุณาให้รายละเอียด พร้อมแนบหลักฐาน)*

1. สิ่งพิมพ์ หรือสื่อทั่วไป

   ☐ **หนังสือพิมพ์** ☐ **วารสาร** ☐ **โทรทัศน์** ☐ **วิทยุ** ☐ **เว็บไซต์** ☐ **คู่มือ/แผ่นพับ** ☐ **จัดประชุม/อบรม** ☐ **อื่น ๆ**

   ...ไม่มี.....

   .....

2. สิ่งพิมพ์ทางวิชาการ *(วารสาร, การประชุม ให้ระบุรายละเอียดแบบการเขียนเอกสารอ้างอิง เพื่อการค้นหาซึ่งควรประกอบด้วย ชื่อผู้แต่ง ชื่อเรื่อง แหล่งพิมพ์ ปี พ.ศ. (ค.ศ.) ฉบับที่ หน้า)*

   ...ไม่มี.....

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Abstract

Project Code : MRG5580103

Project Title : Microbiological and chemical changes of squid (*Loligo formosana*) preserved by combined methods during refrigerated storage

Investigator : Asst. Dr.Punnanee Sumpavapol

Faculty of Agro-Industry, Prince of Songkla University

E-mail Address : punnanee.s@psu.ac.th

Project Period : 2 years

Squids are the main marine animals in the world cephalopod fishery production. During handling or storage, squids undergo changes in texture, flavor as well as color, leading to less acceptability. Quality changes of squids are generally dependent on storage conditions including storage temperature and time. Generally, the rapid growth of microorganisms is associated with the spoilage of squid. However, there is very little information on the role of specific spoilage microorganisms in the quality loss of squid during postmortem handling and storage. Therefore, this study aims to identify the major spoilage microorganisms in squid during refrigerated storage. And further, elucidate the combination method to prolong the shelf-life of squid keep at refrigerated temperature. All squid samples (1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning) were stored in a refrigerator (4°C) for 14 days. During storage, it was found that microbial count, TVB, TMA contents, pH and color of squid undergo changes and spoiled within 2 days of storage. *Aeromonas aeroginosa* was found to be a specific spoilage organism of squid due to it accelerates the quality changes of squid compared to other identified microorganisms. Moreover, squid treated with citric acid could retard the shelf-life of squid from 2 to 4 days while MAP could extend the shelf-life from 2 to 6 days. A combination of citric acid treated with MAP could extend the shelf-life of squid (whole squids, squids with evisceration and squids with evisceration and skinning) from 2 to 8 days of storage. Further identified the associate of *Aeromonas aeroginosa* and other microorganisms caused spoilage of squid could be fruitfully to seafood industries

Keywords : Squid; Refrigerated storage; Microbiological change; Chemical change

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Acknowledgement

The research project " Microbiological and chemical changes of squid (*Loligo fomosana*) preserved by combined methods during refrigerated storage" is funded by Thailand Research Fund (TRF), Office of the Higher Education Commission (OHEC) and Prince of Songkla University (PSU) for the fiscal year of 2012. The researcher would like to thank you very much for this opportunity.

This research was successful with the support from Faculty of Agro-Industry, Prince of Songkla University for places, tools and research equipment. The researcher would like to thank you very much.

Punnanee Sumpavapol

Researcher

Abstract

Squids are the main marine animals in the world cephalopod fishery production. During handling or storage, squids undergo changes in texture, flavor as well as color, leading to less acceptability. Quality changes of squids are generally dependent on storage conditions including storage temperature and time. Generally, the rapid growth of microorganisms is associated with the spoilage of squid. However, there is very little information on the role of specific spoilage microorganisms in the quality loss of squid during postmortem handling and storage. Therefore, this study aims to identify the major spoilage microorganisms in squid during refrigerated storage. And further, elucidate the combination method to prolong the shelf-life of squid keep at refrigerated temperature. All squid samples (1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning) were stored in a refrigerator (4°C) for 14 days. During storage, it was found that microbial count, TVB, TMA contents, pH and color of squid undergo changes and spoiled within 2 days of storage. *Aeromonas aeroginosa* was found to be a specific spoilage organism of squid due to it accelerates the quality changes of squid compared to other identified microorganisms. Moreover, squid treated with citric acid could retard the shelf-life of squid from 2 to 4 days while MAP could extend the shelf-life from 2 to 6 days. A combination of citric acid treated with MAP could extend the shelf-life of squid (whole squids, squids with evisceration and squids with evisceration and skinning) from 2 to 8 days of storage. Further identified the associate of *Aeromonas aeroginosa* and other microorganisms caused spoilage of squid could be fruitfully to seafood industries.

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บทคัดย่อ

หมึกเป็นสัตว์น้ำเศรษฐกิจชนิดหนึ่งของของโลก ในระหว่างการจัดการหรือการเก็บรักษาปลาหมึกจะมีการเปลี่ยนแปลงของเนื้อสัมผัส รสชาติ และสี จนไม่เป็นที่ยอมรับอย่างรวดเร็ว การเปลี่ยนแปลงคุณภาพของปลาหมึกโดยทั่วไปขึ้นอยู่กับสภาพการเก็บรักษา รวมถึงอุณหภูมิ และเวลาในการเก็บรักษา โดยทั่วไปการเจริญเติบโตของจุลินทรีย์อย่างรวดเร็วส่งผลให้เกิดการเน่าเสียของหมึก อย่างไรก็ตาม มีข้อมูลเพียงเล็กน้อยเกี่ยวกับบทบาทของจุลินทรีย์ที่ทำให้เกิดการเน่าเสีย โดยเฉพาะในการสูญเสียคุณภาพของปลาหมึกในระหว่างการจัดการและการเก็บรักษาหลังการจับ การศึกษานี้จึงมีวัตถุประสงค์เพื่อระบุชนิดของจุลินทรีย์ที่ทำให้เกิดการเน่าเสียของหมึกระหว่างการเก็บรักษาในอุณหภูมิตู้เย็น และยังศึกษาการถนอมอาหารแบบร่วมเพื่อยืดอายุการเก็บของหมึกที่อุณหภูมิตู้เย็น โดยตัวอย่างหมึกทั้งหมดได้แก่ (1) หมึกทั้งตัว 2) หมึกควักไส้ และ 3) หมึกควักไส้และลอกหนัง ถูกเก็บไว้ในตู้เย็น (4°C) เป็นเวลา 14 วัน ผลการศึกษาพบว่า ในระหว่างการเก็บรักษาพบจำนวนจุลินทรีย์ทั้งหมด ค่า TVB และ TMA pH และสีของหมึกมีการเปลี่ยนแปลงอย่างรวดเร็ว และเกิดการเน่าเสียภายในเวลา 2 วันหลังการเก็บรักษา โดยเชื้อจุลินทรีย์ *Aeromonas aeroginosa* พบว่าเป็นสาเหตุสำคัญของการเน่าเสียของหมึก โดยไปเร่งการเปลี่ยนแปลงคุณภาพของปลาหมึกเมื่อเทียบกับจุลินทรีย์อื่น ๆ ยิ่งไปกว่านั้น หมึกที่ใช้สารต้านจุลินทรีย์ (กรดซิตริก) สามารถชะลออายุการเก็บของปลาหมึกได้จาก 2 เป็น 4 วัน ในขณะที่การบรรจุในสภาวะดัดแปรบรรยากาศ (MAP) สามารถยืดอายุการเก็บได้จาก 2 เป็น 6 วัน ผลของการใช้กรดซิตริกร่วมกับการบรรจุในสภาวะดัดแปรบรรยากาศสามารถยืดอายุการเก็บของหมึก (หมึกทั้งตัว หมึกควักไส้ และหมึกควักไส้และลอกหนัง) จาก 2 เป็น 8 วัน การศึกษาเพิ่มเติมถึงผลของเชื้อ *Aeromonas aeroginosa* กับเชื้อจุลินทรีย์อื่น ๆ ที่ทำใหหมึกเน่าเสียจะมีประโยชน์อย่างยิ่งต่ออุตสาหกรรมอาหารทะเล

คำสำคัญ : หมึก, การเก็บรักษาที่อุณหภูมิตู้เย็น, การเปลี่ยนแปลงทางจุลินทรีย์, การเปลี่ยนแปลงทางเคมี

Executive Summary

Squids are the main marine animals in the world cephalopod fishery production. During handling or storage, squids undergo changes in texture, flavor as well as color, leading to less acceptability. Moreover, pink discoloration in squid mantle during handling or storage is the problem, causing the losses in quality and acceptability. Quality changes of squids are generally dependent on storage condition including storage temperature and time. Generally, the rapid growth of microorganisms is associated with the spoilage of squid. However, there is very little information on the role of specific spoilage microorganisms in the quality loss of squid during postmortem handling and storage. Therefore, this study aims to identify the major spoilage microorganisms in squid during refrigerated storage. And further, elucidate the combination method to prolong the shelf-life of squid keep at refrigerated temperature. All squid samples (1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning) were stored in a refrigerator (4°C) for 14 days. During storage, it was found that microbial count, TVB, TMA contents, pH and color of squid undergo changes and spoiled within 2 days of storage. *Aeromonas aeroginosa* was found to be a specific spoilage organism of squid due to it accelerates the quality changes of squid compared to other identified microorganisms. Moreover, squid treated with citric acid could retard the shelf-life of squid from 2 to 4 days while MAP could extend the shelf-life from 2 to 6 days. A combination of citric acid treated with MAP could extend the shelf-life of squid (whole squids, squids with evisceration and squids with evisceration and skinning) from 2 to 8 days of storage. Further identified the associate of *Aeromonas aeroginosa* and other microorganisms caused spoilage of squid could be fruitfully to seafood industries.

Introduction

The exploitation of cephalopods has increased in recent years as a consequence of the decline stocks of commonly exploited fish species. The world demand for cephalopods increased greater than that related for marine fishes, indicating a specific demand for these marine animals. Squids are the main marine animals in the world cephalopod fishery production, mainly due to their excellent sensory properties (Sikorski and Kolodziejska, 1986).

Loligo formosana (*L. chinensis*) is the economically important squid in the Southeast Asia. This squid is caught along Western Pacific Southern Japan to Northern and Northeastern Australia. In Thailand, *L. formosana* has been caught along the Gulf of Thailand and Andaman sea. It is very important species of Thailand and its common name is “Splendid squid” (Department of Fisheries, 2006). Among 10 kinds of squids caught in Thailand, *L. formosana* is caught for about 12.65% of the total squid catch (Department of Fisheries, 2006).

During handling or storage, cephalopods undergo changes in texture, flavor and color. Quality changes of squids are generally dependent on storage condition (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Lapa-Guimaraes *et al*., 2002; Lapa-Guimaraes *et al*., 2005). The pH, total volatile base (TVB) and polyamine of squid increased with increasing storage temperatures (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Sungsri-in *et al*., 2011).

Early studies of seafood microbiology indicated that only part of the spoilage microflora participated in the spoilage process (Huss *et al*., 1974; Castell and Anderson, 1948). Nevertheless, the recent establishment of the specific spoilage organism (SSO) concept (Dalgaard, 2000) has contributed significantly to our understanding of seafood spoilage. The SSOs are typically present in low numbers and constitute only a very small fraction of the microflora on newly processed seafood. Different SSOs are found in different seafoods (Dalgaard, 2000). Identification of an SSO relies on comparison of the sensory and chemical characteristics of spoiled products with those of the spoilage microflora. The qualitative ability to produce off-odors (spoilage potential) and the quantitative ability to produce spoilage metabolites (spoilage activity) are essential in the identification of an SSO (Dalgaard, 2000). Quantitative comparison of the production of trimethylamine (TMA), biogenic amines or volatile amines in products and that mediated by bacterial isolates grown in model substrate are useful for the identification of SSOs (Jorgensen *et al*., 2000; Dalgaard, 1995; Koutsoumanis and Nychas, 2000). Comparison of the chemical profiles of spoiled seafoods and of the metabolites produced by potential spoilage organisms has only been used to a limited extent in

identification of SSOs. The spoilage domain has been defined as the range of conditions (pH, temperature, water activity and atmosphere) under which an SSO can grow and produce spoilage metabolites (Dalgaard, 2000). Therefore, the identification of SSOs and their spoilage domains could facilitate the development of methods to determine, predict and extend the shelf life of squid.

To retard the spoilage of fish and fish products, hurdle preservation technology has been implemented. Some of these hurdles include low storage temperatures, thermal treatments, water activity, salt, preservatives, pH and other techniques including vacuum packaging (VP) and modified atmosphere packaging (MAP), bioconservation, bacteriocin, etc. (Leistner and Gorris, 1995). Hurdle technology usually works by combining more than one approach. These approaches can be thought as "hurdles" for spoilage microorganisms. The appropriate combination of hurdles can lower the spoilage of squid, thereby maintaining the quality and acceptability of squid during the storage.

Objectives

1. To monitor the microbiological and chemical changes of squid during refrigerated storage
2. To identify the specific spoilage organisms of squid during refrigerated storage
3. To study the impact of some antimicrobial agents on the microbiological and chemical changes of squid during refrigerated storage
4. To study the effect of packaging atmospheres on the microbiological and chemical changes of squid during refrigerated storage
5. To investigate microbiological and chemical changes of squid preserved by combined methods during refrigerated storage

Literature Review

Squid

Squid belongs to class Cephalopod, which forms part of phylum Mollusca and subclass Coleoideas. All species of squid have adapted to different environments, from coastal waters to oceanic regions, from surface waters to deep-sea zones, and from tropical seas to polar regions (Okuzumi and Fujii, 2000). The part generally termed the body is the mantle. The arms are at the front, while the fins are at the rear. The surface with the shell is the dorsal surface. The surface with the funnel is the ventral surface. The mantle dorsal surface has more chromatophores than the ventral surface.

The composition and general properties of squid vary depending on the species, the season, the growth stage and other factors. According to the 4th Amended Japanese Standard Food Content, the edible parts of squid contained 81.8% water, 15.6% protein, 1.0% fat and 1.5% ash. Lapa-Guimaraes *et al*. (2005) reported that squid (*L. plei*) contained 74.2% water, 4.4% protein, 2.0% fat and 1.7% ash. Squid protein is composed of 80% myofibrillar protein, 12-20% sarcoplasmic protein and 2-3% collagen. In addition, squid muscle contains non-protein nitrogenous compounds at approximately 37% of total nitrogen content (Sugiyama *et al*., 1989). Those include free amino acids, betaines, trimethylamine oxide (TMAO), nucleotides, octopine and trimethylamine (Okuzumi and Fujii, 2000). Amino acids, especially essential amino acids, determine primarily the nutritive food value; however, it can be used by invading bacteria, producing ammonia and putritive smell (Kreuzer, 1984). The lipid content of raw squid is about 1.0-2.0% comprising 62-84% phospholipids, 15-20% sterols and 0.8-3.2% triglycerides. Phospholipids in squid have lecithin and phosphotidyl-ethylnolamine as the major compounds (Thanonkaew *et al*., 2006). Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and palmitic acid are the dominant fatty acids in squid (Okuzumi and Fujii, 2000).

Spoilage organisms in seafoods

Generally, 25% of all food is lost post-harvest owing to microbial activity (Baird-Parker, 2000). An improved science-based understanding of the growth and activity of spoilage microorganisms in seafood and other foods is crucial for the development of preservation techniques and subsequent reduction of losses due to spoilage (Dalgaard, 2000).

Microbial ecology of seafoods

During storage, the microflora changes occur depending upon the abilities of the microorganisms to tolerate the preservation conditions. Gram-negative, fermentative bacteria (such as *Vibrionaceae*) spoil unpreserved fish, whereas psychrotolerant Gram-negative bacteria (*Pseudomonas* spp. and *Shewanella* spp.) grow on chilled fish (Gram and Huss, 2000). CO_2 was able to inhibit respiratory organisms and selects for *Photobacterium phosphoreum* and lactic acid bacteria (LAB) (Dalgaard, 2000). Respiratory Gram-negative bacteria are typically inhibited in fish products preserved by the addition of low levels of NaCl, a slight acidification and chilled storage in vacuum-packs. Under these conditions, the microflora typically becomes dominated by LAB (*Lactobacillus* and *Carnobacterium*) with an association of Gram-negative fermentative bacteria such as *P. phosphoreum* and psychrotrophic Enterobacteriaceae (Truelstrup *et al*., 1995; Jorgensen *et al*., 2000; Leroi *et al*., 1998). Increasing the 'preservation pressure', for example, by acidification or the addition of preservatives like sorbate and benzoate, in the so-called semipreserved seafood (e.g. marinated herring), allows the growth of lactobacilli and yeasts (Fernandez-Segovia *et al*., 2007).

Specific spoilage organism

Different SSOs are found in different seafoods and may be a single species. *S. putrefaciens* was found in iced marine fish, wherever *Pseudomonas* spp. was dominant in iced freshwater fish (Gram and Dalgaard, 2002). *P. phosphoreum* was the specific spoilage bacteria in CO_2-packed chilled fish (Gram and Dalgaard, 2002). Spoilage is also dependent on the climatic and storage conditions, the type of fish and even of place in which the fish was harvested (Drosinos and Nychas, 1996; Gram and Huss, 1996). *S. putrefaciens*, *Pseudoalteromonas* spp. and *Pseudomonas* spp. are spoilage bacteria of gutted squid stored in ice (Paarup *et al*., 2002). The major spoilage flora in cuttlefish (*Sepia officinalis*) and short fin (*Illex coindetii*) storage in ice was *Pseudomonas* (Vaz-Pires *et al*., 2008).

Microbial metabolites and seafood spoilage

Fish contains little carbohydrate but typically has a high content of free amino acids. Many fish species contain TMAO. The seafood SSOs produce ammonia, biogenic amines, organic acids and sulfur compounds from amino acids, hypoxantine from ATP degradation products and acetate from lactate. TMA is produced by some bacteria capable of using TMAO in anaerobic respiration (Shewan, 1977; Chinivasagam *et al*., 1998; Olafsdottir and Fleurence, 1998; Koutsoumanis and Nychas, 1999; Joffraud *et al*., 2001; Jorgensen *et al*., 2001). Many microbial metabolites produced in seafood are similar to those observed in meat and poultry products (Dainty and Mackey, 1992; Nychas *et al*., 1998). However, in seafood spoilage, TMA

in particular contributes to the characteristic ammonia-like and 'fishy' off-flavours. *Aeromonas* spp., psychrotolerant Enterobacteriaceae, *P. phosphoreum*, *S. putrefaciens*-like organisms and *Vibrio* spp. can all reduce TMAO to TMA. Several compounds produced by microbial metabolism during spoilage are given in Table 2.

Table 1. Bacterial spoilage compounds.

Specific spoilage organism	Spoilage compounds
Shewanella putrefaciens	TMA, H_2S, CH_3SH, $(CH_3)_2S$, HX
Photobacterium phosphoreum	TMA, HX
Pseudomonas spp.	Ketones, aldehydes, esters, non-H_2S sulphides
Vibrionacaea	TMA, H_2S
Aerobic spoilers	NH_3, acetic, butyric and propionic acid

TMA = trimethylamine, H_2S = hydrogen sulphide, CH_3SH = methylmercarptan, $(CH_3)_2S$ = dimethylsulphide, HX = hypoxanthine, NH_3 = ammonia

Source: Church (1998)

Some spoilage metabolites can be used as quality indices. Compared with microbiological methods, which are slow, chemical analyses may be significantly faster. For some compounds, the measurable concentrations are not present until close to spoilage. Classical single-compound quality index (SCQI) for seafood includes total volatile nitrogen (TVN), TMA and hypoxantine. K-values) and biogenic amine content have also been used as quality indices (Dalgaard, 2000). Multiple-compound quality indices (MCQI), in which combinations of several metabolites are identified by statistical methods, have been introduced and correlate better with sensory properties and/or shelf life in some products (Jorgensen *et al*., 2000; Jorgensen *et al*., 2001; Leroi *et al*., 2001).

Interactions between spoilage bacteria

Spoilage bacteria are selected primarily as a result of the physical and chemical conditions in the products; however, seafood spoilage obviously involves growth of the microorganisms to high numbers (>10^6-10^7 cfu/g) and the interaction (antagonism or symbiosis) between different groups of microorganisms may influence their growth and metabolism. Despite the nutrient richness of fish muscle, the environment becomes iron-limited and siderophores are produced during bacterial growth (Gram and Melchiorsen, 1996). The high iron-binding capacity of the pseudomonas siderophores may cause this bacterial group to be

positively selected (Gram and Melchiorsen, 1996). LAB inhibits growth of other bacteria due to the formation of lactic acid and bacteriocins or by competition for nutrients and this may contribute to their selection during spoilage of lightly preserved seafood products (Jorgensen *et al*., 2000). Lactobacilli and Enterobacteriaceae might interact during spoilage of lightly preserved fish products. LAB may degrade arginine to ornithine, which is then degraded by the Enterobacteriaceae to putrescine. This results in 10-15 fold higher levels of putrescine than that produced by Enterobacteriaceae growing alone in the absence of LAB (Jorgensen *et al*., 2000).

Preservation technology

Fish and fish products can be preserved by different methods, when the microbial growth can be impeded. Different preservation methods showed different efficiency in shelf-life extension.

Low storage temperature

Icing has been used as a common chilling method to maintain the quality of squids. Squid (*L. plei*) quality markedly decreased after 7 days of storage in both contact and non-contact ice. However, the storage methods did not affect the development of bacteria counts, in which both treatments were below 10^6 cfu/g of muscle after 16 days of storage (Lapa-Guimaraes *et al*., 2002). Chemical and microbial changes in squid muscle (*Loligo plei*) during iced storage by contact ice and non-contact ice were compared by Lapa-Guimaraes *et al*. (2005). TMA and TVB contents increased during the first day of storage in the non-contact ice treatment, related with increasing psychrotrophic bacteria count. Free tryptophan and urea production by microorganisms markedly increased during the storage. Sample stored by contact ice had the increase in TMA and TVB after day 9 and no increase was observed in free tryptophan and urea contents (Lapa-Guimaraes *et al*., 2005). In general, the non protein nitrogen (NPN) continuously decreased in both squid and cuttlefish during storage due to leaching process.

Evisceration of squid is another means to extend the shelf-life along with iced storage. The gutting of *Todaropsis eblanae* extended shelf-life by approximately two days and reduced the production of ammonia and TMA. Agmatine production occurs during the early storage stages. This amine seems to be an excellent freshness indicator (Paarup *et al*., 2002). *P. phosphoreum* may contribute to spoilage through its activity in the digestive gland, followed by diffusion of volatile compounds and amines to the mantle (Moral *et al*., 2002).

Autolysis contributing to quality may be enhanced by the presence of proteases of microbial origin. The autolytic activity of squid (*L. vulgaris*) was found on day 4 of chilled

storage and a drop was seen at day 7. These fluctuations were attributed to variations in the endogenous muscle proteases and their activities, which are affected by the post-rigor condition and softening of the muscle (Gomez-Guillen *et al*., 2003). Autolysis increased sharply at day 10 of chilled storage. The microbial protease activity may be significant after 10 day of iced storage, when muscle decomposition becomes apparent (Gomez-Guillen *et al*., 2003). The microbial action is less evident than autolysis in cephalopods until sensory rejection (Vaz-Pires *et al*., 2008). The autolysis or activity of endogenous enzymes was the main responsible for the change in sensory attributes (Vaz-Pires *et al*., 2008).

Modified atmosphere packaging (MAP)

In recent years, the interest in modified atmosphere packaged foods has increased and establishment of storage conditions to assure product safety and sufficient shelf life for distribution is an important issue (Gould, 2000). Fish stored under MAP is considered a fresh product. It is well established that CO_2 inhibits normal fish spoilage flora. Microbial growth rates after storage under MAP are lower than those observed on fish spoilage in air (Stenstrom and Molin, 1990). Carbon dioxide causes a decrease in intra- and extracellular pH and interferes with the cellular metabolism (Dixon and Kell, 1989).

Microbial safety of fish stored under MAP fish has been studied (Silliker and Wolfe, 1980; Reddy *et al*., 1992; Kimura and Murakami, 1993; Kirov, 2001). Silverstsvik *et al*. (2002) reported that storage of products under MAP lowered the risk from pathogens such as *Salmonella, Staphylococcus, Clostridium perfringens* and *Enterococcus.* MAP containing high levels of CO_2 effectively inhibits growth of *Aeromonas hydrophila* particularly at low temperatures (Ingham, 1990; Davies and Slade, 1995; Devlieghere *et al*., 2000). Ingham and Potter (1988) reported growth of *A. hydrophila* in surimis made from Atlantic Pollock store under MAP 36% CO_2 / 51% N_2 / 13% O_2 at 8 days at 4°C. Mejholm *et al*. (2005) evaluated the growth of *Listeria monocytogenes* in cooked peeled shrimps in MAP and found that potential growth of this organism limited shelf-life of the product more effectively than the growth of spoilage bacteria and non-proteolytic *C. botulinum*. Facultative anaerobic microorganisms and aerobic pathogens are resistant to the antimicrobial effects of CO_2 (Wolfe, 1980). However, the pathogen of major concern when packaging fish under anaerobic conditions has been psychrotrophic *C. botulinum*.

The ideal CO_2 concentration in MAP depends on the fish species (Soccol and Oetterrer, 2003) and different gas atmospheres have been used for fish products. Gas mixtures presenting 40% CO_2 / 30% N_2 / 30% O_2 have been recommended for low fat fish and a 40-60% CO_2 mixture in equilibrium with N_2 has been recommended for fatty fish (Cann *et al*., 1984;

Soccol and Oetterrer, 2003). Optimal MAP condition for fish and fish products varies with species, form, etc. Gas mixtures containing 60% CO_2 / 40% O_2 for cod (Stenstrom, 1985; Debevere and Boskou, 1996), were suitable for whole gutted hake (Ruiz-Capillas and Moral, 2001) and Tilapia (Soccol *et al*., 2005). Gas mixtures of 40% CO_2 / 60% O_2 for sole (Lopez-Galvez *et al*., 1998), 40% CO_2 / 30% N_2 / 30% O_2 for cod (Cann *et al*., 1983), Haddock (Drosinos *et al*., 1997) and seabream (Goulas and Kontominas, 2007), 60% CO_2 / 40% N_2 (Cann *et al*., 1984) and 40% CO_2 / 60% N_2 (Randell *et al*., 1995) for trout and herring have been reported. Stefansson and Lauzon (1999) reported that the use of O_2 with CO_2 was preferable to N_2 as a filling gas, providing a slightly longer shelf-life for these lean fishes. Arashishar *et al*. (2004) found that although 40% CO_2 / 30% N_2 / 30% O_2 is recommended for lean fish, it has been found to be the worst choice for freshwater rainbow trout stored at 4°C.

Antimicrobial agents

Antimicrobial agents are substances added, which are able to preserve food products. Organic acids and their salts (acetic, citric, lactic, propionic and sorbic) exert antimicrobial activity. They have been traditionally used as food preservatives and are generally recognized as safe substances (GRAS) approved as food additives by E.C., FAO/WHO and FDA (Surekha and Reddy, 2000).

Sorbic acid and its salts have several advantages as food preservatives. They have been known to also inhibit a wide range of bacteria, particularly aerobic catalase-positive organisms (Thomas, 2000). Effective concentrations do not normally alter product taste or odor. These preservatives are also considered harmless (Thomas, 2000). Potassium salt is commonly used because it is more stable. Furthermore, its greater solubility extends the use of sorbate to solutions appropriate for dipping and spraying (Thomas, 2000). Potassium sorbate has been extensively investigated as an antimicrobial agent for use in fish to extend its shelf life and inhibit the growth of pathogens. Moreover, effective concentrations do not affect sensory characteristics of products (Dalgaard *et al*., 1998; Fey and Regenstein, 1982; Reddy *et al*., 1992; Fernandez-Segovia *et al*., 2007). Dalgaard *et al*. (1998) demonstrated that potassium sorbate markedly reduced growth of *P. phosphoreum*, identified as the specific spoilage organism in cod fillet (Dalgaard, 1995; Dalgaard *et al*., 1997) and Salmon (Emborg *et al*., 2002) stored under MAP during chilled storage.

Citric acid is a weak organic acid serving as a natural preservative in various products (Fernandez-Segovia *et al*., 2006). This organic acid is soluble in lipids in their undissociated forms, which allows them to cross the microbial membrane into the microbial cytoplasm, where the acids tend to dissociate and deliver hydrogen ions and a particular anion (Booth, 1985;

Booth and Kroll, 1989). As a result, microorganisms are forced to export the excess hydrogen ion to maintain a physiological pH inside the cell, which is an energy-depleting process that limits bacterial growth. Otherwise, the excess hydrogen ions in the cytoplasm may cause the pH to decrease to levels that are incompatible with bacterial growth (Gould, 1996). Moreover, citric acid is well-known chelator and acidulant in biological systems, which is especially beneficial for minced fish (Stodolnik *et al*., 1992), fish fillets (Badii and Howell, 2002; Pourashouri *et al*., 2009) and whole fish (Aubourg *et al*., 2004).

Moreover, potassium sorbate and citric acid have been used in combination with VP or MAP to extend shelf-life of fish products stored at refrigerated temperature (Dalgaard *et al*., 1998; Fey and Regenstein, 1982; Reddy *et al*., 1992; Fernandez-Segovia *et al*., 2007).

Methodology

1. Squid collection and preparation

Squids (*L. formosana*) with the sizes of 6-10 squids/kg, caught by cast net from Songkhla coast along the Gulf of Thailand and off loaded after 24 h of capture, will be purchased from a dock in Songkhla province. The squids will be placed in the insulated boxes containing ice with a squid/ice ratio of 1:2 (w/w) and transported to the laboratory within 1 h. Upon arrival, the whole squids will be divided into three groups as follows: 1) whole squid 2) squid with evisceration and 3) squid with evisceration and skinning. All samples will be washed with cold water (4°C) and kept on ice during the preparation.

2. Microbiological and chemical changes of squid during refrigerated storage

2.1 Storage of prepared squids

All squid samples will be placed on polystyrene trays, cover with plastic film and will be stored in a refrigerator (4°C). During storage of 14 days, the samples will be randomly taken for analyses every 2 days. The edible portion (mantle and tentacle) will be collected for analysis.

2.2 Microbiological analysis

A 25 g portion of samples will be dissected aseptically, mixed with 225 ml of peptone water and homogenized in a stomacher for 3 min (Seward Medical, London, UK). Ten-fold serial dilution from the microbial extracts will be prepared in peptone water. Sample (0.1 ml) of serial dilutions of squid homogenates will be spread on the surfaces of appropriate media in duplicate petri dishes.

For the enumeration of mesophilic and psychrotrophic bacteria, plate count agar will be incubated at 37°C and 10°C for 2 and 10 days, respectively.

To enumerate pseudomonas, cetrimide-fusidin-cephaloridine agar will be incubated at 20°C for 3 days. *Aeromonas* spp. will be counted on starch ampicillin agar containing 10 μg/ml of ampicillin at 28°C for 48 h based on the method of Palumbo *et al*. (1985) and amylase positive yellow to honey-colored colonies will be scored as presumptive *Aeromonas* spp.

For Enterobacteriaceae and H_2S-producing bacteria (including *Shewanella putrefaciens*) enumeration, one ml sample will be inoculated into 10 ml of molted (45°C) violet red bile glucose agar (VRBGA) and iron agar, respectively. After setting, a 10 ml overlay of molten medium will be added. VRBGA plates will be incubated at 30°C for 24 h. The large

colonies with purple haloes will be counted. Iron agar plates will be incubated at 20°C for 5 days; black colonies formed by the production of H_2S will be enumerated after 2-3 d (Gram *et al*., 1987). *Enterococci* numbers will be estimated on KF Streptococci agar. Five typical *Enterococcus* colonies will be identified by checking growth at 45±1°C and growth in dextrose azide broth containing 6.5% sodium chloride incubate at 35±2°C and will be confirmed by biochemical tests as described by APHA (1998).

Sulphite-reducing clostridia will be enumerated on tryptose sulphite cycloserine (TSC) agar, overlaid with the same medium and incubated anaerobically in anaerobic jar at 30°C for 5 days (Mossel, 1987). All plates will be examed for typical colony types and morphological characteristics associated with each growth medium. Selected colonies will be checked routinely by Gram staining and microscopic examination of smear. LAB counts will be estimated on MRS agar incubates at 30°C for 2 days and typical colonies will be confirmed by catalase test and Gram staining.

2.3 Chemical analysis and color measurement

TVB and TMA contents will be determined following the method of Conway and Byrne (1936). pH of sample will be measured according to the method of Benjakul *et al*. (1997).

Color of squid mantle after skin removal will be measured using a colorimeter (HunterLab, Model ColorFlex) and reported in CIE system color profile of L* (lightness), a* (redness/greenness) and b* (yellowness/blueness). For each sample, the color will be measured at six positions of the mantle.

3. Identification of the specific spoilage organisms of squid during refrigerated storage

3.1 Identification of the strains

Five to ten selected colonies with distinct colony morphology, representing the major population of bacteria from each medium used above, will be re-streaked to obtain the pure culture. Pure collection will be placed in appropriated media at 4°C during analysis or kept at -80°C as a stock culture. All selected isolates will be subjected to phenotypic characterization and genotypic characterization by 16S rRNA gene analysis.

4. Study on the impact of selected isolate on quality changes of squid during refrigerated storage

4.1 Preparation of selected isolates

Stock culture of all identified isolates will be resuscitated in culture medium and incubated at appropriate temperature for 1-2 days. Then growth culture will be transferred to

culture medium and incubated at appropriate temperature for another 1-2 days. Cell culture will be collected by centrifugation at 9000 X g for 10 min at 4°C. Pellet will be suspended in phosphate buffer saline (pH 7.4) to obtain a final cell concentration between 10^4 and 10^6 cfu/g.

4.2 Preparation of squid with inoculation

To minimize the interference of natural microorganisms of squid with inoculated microorganism, squid with evisceration and skinning will be washed in sterile cold water for twice and 500 g sample will be transferred to sterile plastic tray. Aliquot of inoculate will be transferred to a surface of squid, mixed thoroughly, covered with plastic film and kept in the refrigerator (4°C). During storage of 14 days, the samples will be randomly taken for cell enumeration and chemical analyses every 2 days as previously described.

4.3 Selected of SSO

Based on chemical changes of squid inoculated with different isolates, those (3-5 isolates) causing the highest rate of decomposition as indicated by the highest formation of TVB and TMA content will be identified as specific spoilage organism; SSO.

5. Study on the effects of antimicrobial agents and packing atmosphere on quality changes of squid during refrigerated storage

5.1 Effect of antimicrobial agents

For all squids (section 10.1), each sample will be divided into 3 groups. First group will be submerged in 3% NaCl solution containing 0.45% potassium sorbate (KS) while the second group will be treated with 3% NaCl solution containing 0.2% citric acid for 10 min. The third group without any treatment will be used as the control. All samples will be placed in the plastic tray, covered with film and kept in a refrigerator. The temperature of squid stored in refrigerator will be maintained at 4°C. During storage of 14 days, the samples will be randomly taken for psychrotrophic bacterial and selected SSO (section 10.3 and 10.4) count and chemical analyses every 2 days.

5.2 Effect of packaging atmospheres

For each squid sample (section 10.1), it will be divided into 3 groups. Three packaging atmospheres will be used as follows: 1) air 2) vacuum and 3) MAP (60% CO_2 / 30% N_2 / 10% O_2). All samples will be packed in polyamide/polyethylene (PA/PE) 20/70 pouches with 90 μm in thickness. During storage of 14 days in refrigerator at 4°C, the samples will be randomly taken for analyses every 2 days as described in section 10.5.1.

5.3 Effect of combined methods

All squid samples (section 10.1) will be inoculated with the selected SSO (section 10.4.3) at a level of 10^6 cfu/g in the presence or absence of pathogenic bacteria (*Escherichia coli*) at a level of 10^3 cfu/g (a maximum limit for chilled squid export). All samples will be treated with antimicrobial agent yielding the highest efficacy in maintaining the quality, followed by packaging under the packaging atmosphere rendering the highest ability in retardation of deterioration. Samples which submerged in 3% NaCl solution without any treatments will be used as the control. During storage of 14 days in a refrigerator at 4°C, the samples will be randomly taken for determination of psychrotrophic bacterial and selected SSO (10.4.3) counts every 2 days. Chemical analyses will be also conducted every 2 days.

6. Statistical analysis

Completely randomized design (CRD) will be used for entire studies. Experiments will be run in triplicate. Data will be subjected to analysis of variance (ANOVA). Comparison of means will be carried out by Duncan's multiple range test. For pair comparison, *T*-test will be used. Statistical analysis will be performed using the statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Result and Discussion

1. Microbiological and chemical changes of squid during refrigerated storage

Microbial count of 1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning increased continuously during the storage up to 14 days ($p < 0.05$) (Figure 1.). No marked difference in mesophilic bacteria, psychrophilic bacteria, *Psuedomonas*, sulphite-producing bacteria, and *Enterobacter* count were found between all samples during storage of 14 days excepting *Aeromonas* and *Enterococci* count. For all squid samples, the higher number of psychrophilic bacteria, *Psuedomonas*, *Aeromonas* and sulphite-producing bacteria were noticeable when the storage time was greater than 4 days. For example, PBC increased from 4 to 8 Log cfu/g after 4 days of storage (Figure 1A). The results indicated that storage at refrigerated temperature could not retard the growth of microorganisms of squids, squids with evisceration and squid with evisceration and skinning and the shelf-life of all samples was shorter than 2 days. Lapa-Guimaraes *et al*. (2005) reported that psychrophillic bacteria count of squid at 1 day after catch was equal to 8×10^2 cfu/g while commercialized squid contain 1×10^4 cfu/g at the beginning of storage in ice.

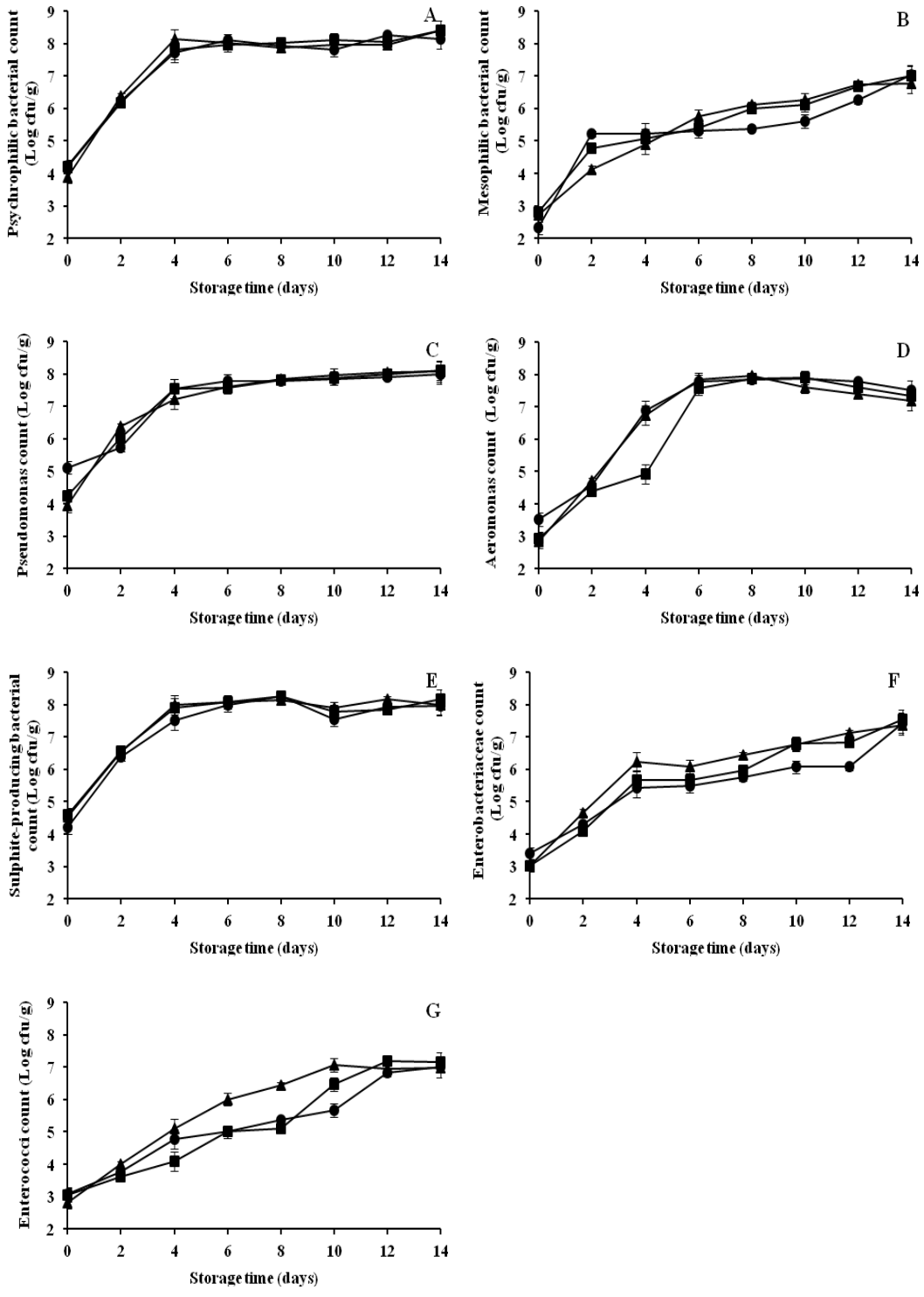

Figure 1. Psychrophilic bacteria, mesophilic bacteria, *Pseudomonas*, *Aeromonas*, sulphite-producing bacteria, *Enterobacteriaceae* and Enterococci counts of whole squids (●), squids with evisceration (■) and squids with evisceration and skinning (▲) during refrigerated storage (4°C). Bars represent the standard deviation (n = 3).

Moreover, The increases in microbial count were in agreement with the increases in TVB, TMA and pH (Figure 2.). The initial TVB contents of squids, squids with evisceration and squid with evisceration and skinning were 7.23 ± 0.404, 7.93 ± 0.808 and 7.93 ± 0.808, respectively. Which was lower than those of other cephalopods obtained 24-36 h after capture (Lapa-Guimaraes et a.,, 2005; Vaz-Pires *et al*., 2008; Sungsri-in, 2010). TVB content of all squid increased gradually within the 2-10 days of storage, suggesting the increase in volatile base compounds formed by endogenous or microbial enzymatic systems. This suggested that volatile compounds produced by microorganisms could be accumulated in the squid mantle. TVB content of squid was higher than those of squids with evisceration and squid with evisceration and skinning, especially after 8 days of storage, indicating the higher spoilage of the former associated with spoilage microorganisms with increasing storage time.

TVB has been considered as usefull as a spoilage indicator (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Sungsri-in, 2010). At the same time of storage, especially during 6-14 days, the sample without any treatment (whole squid) had the higher TVB content, compared with those squids with evisceration and skinning. Ohashi *et al*. (1991) reported that the increases of TVB content were greater in squid kept at 10°C that those found in squid store at 0°C and 5°C. TVB include trimethylamine (TMA), dimethylamine (DMA), monomethylamine (MMA) and ammonia (Seibel and Walsh, 2002). At the same time, the samples without evisceration and skinning had the higher TVB content than the evisceration and skinning counterpart. The skin and visera could be a source of contaminated microorganisms. As a consequence, the microbial invation into the mantle could be more pronounced in the sample without evisceration and skinning.

TMA content of squid without evisceration and skinning increased markedly during 8-14 days of storage (Figure 2B). In general, the resullts of TMA changes in squid were similar to those of TVB changes throughout the storage. TMA is produced by the reduction of trimethylamine oxide (TMAO) by TMAO reductase-producing organisms (Adam and Moss, 2000). TMA contents is an indicator of bacterial deterioration in marine products (Seibel and Walsh, 2002). TMA has been found to be related with a loss of freshness of marine fishery products (Alur et al., 1995; Gram and Huss, 1996). The higher TVB and TMA contents were observed in whole squid compare with those evisceration and skinning. Furthermore, skinning and evisceration of squid tended to lower the rate of increase in both TVB and TMA contents during storage.

pH of all squid samples at different time are shown in Fiigure 2C. The initial pH of whole squid squids with evisceration and squid with evisceration and skinning were 6.5, 6.2 and 6.4, respectively. Sungsri-in (2010) reported that an initial pH of squid (*Loligo formosana*) was 6.40 – 6.58. pH of squid samples increased continuously during storage for 14 days. The rate of increase were greater in sample with evisceration and skinning within 2-4 days of storage. The increase in pH is generally associated with the formation of volative bases (Ohashi *et al*., 1991). Yamanaka *et al*. (1987) reported that the rapid increase in pH in common squid (*Todarodes pacificus*) was noticeable when stored at 15°C, compared with at 0 and 3.5°C.

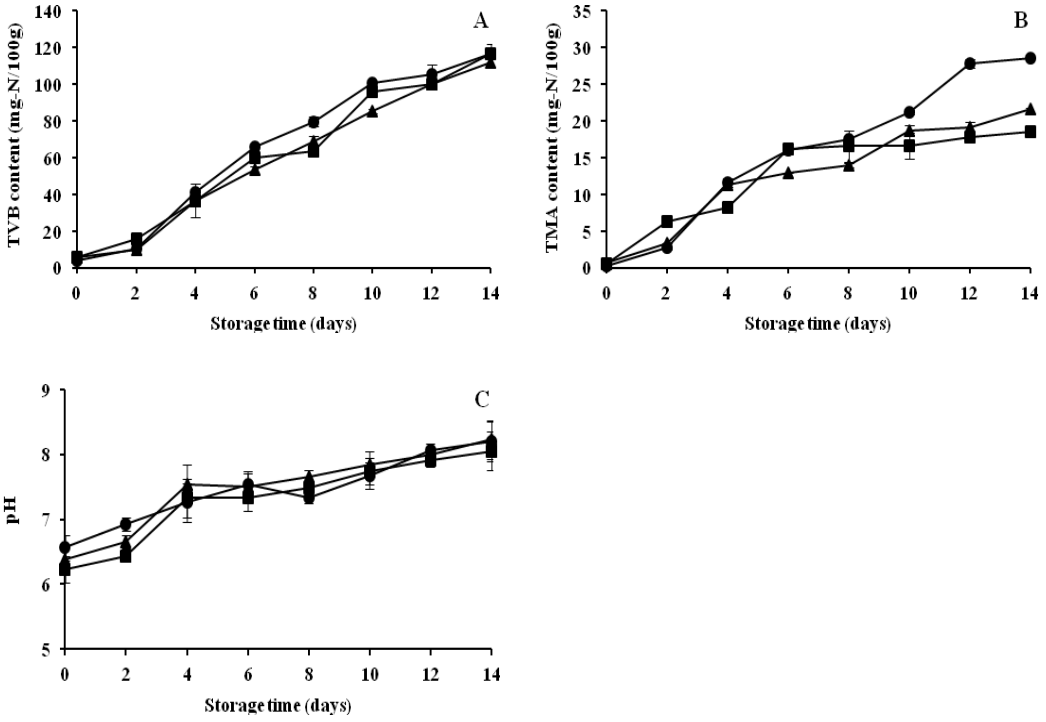

Figure 2. pH, TVB and TMA contents of whole squids (●), squids with evisceration (■) and squids with evisceration and skinning (▲) during refrigerated storage (4°C). Bars represent the standard deviation (*n* = 3).

Moreover, the a* and b*-values of all samples increased up to 14 days of storage, except the a*-values of squids with evisceration and skinning increased. The a*-value of mantle from the squid without skinning for bothe whole squid and squid with evisceration increased continuously with increasing storage time, indicating the formation of pink color on squid mantle (Figure 3B). the increase in a*-values was more pronounced in the samples after 6 days of storage. Lapa-Guimaraes *et al.* (2002) reported that both a* and b*-values in the squid skin and muscle of *Loligo plie* tened to increased during the storage. No changes in a*-values were observed in the mantle of deskinned squid throughot the storage. Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased.

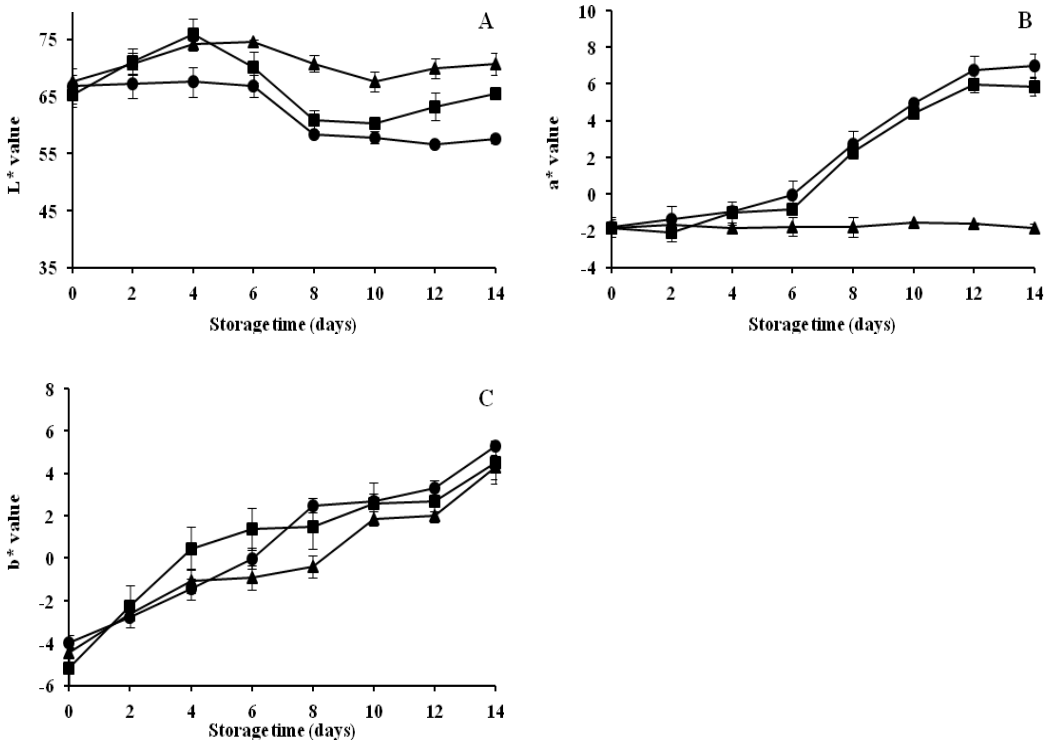

Figure 3. The L*, a* and b*-values of the mantle of whole squids (●), squids with evisceration (■) and squids with evisceration and skinning (▲) during refrigerated storage (4°C). Bars represent the standard deviation (*n* = 3).

For b*-value representing the yellowness of mantle, all samples had the increased in b*-value up to 14 days. Thanonkaew *et al*. (2006) reported that the formation of yellow pigment in squid muscle could be due to nonenzymatic browning reaction occurring between aldehyde lipid oxidation products and the amines on phospholipids head groups. Squid muscle contained phospholipids as the major lipid (Thanonkaew *et al*., 2006). It was noted that the mantle of squid without skinning exhibited the higher increasses in b*-value, compared with the deskinned counterpart.

2. Identification of the specific spoilage organisms of squid during refrigerated storage

Using phenotypic characterization, selected isolates (18) were identified as the member of the Genus *Aeromonas* (3), *Enterobacter* (2), *Enterococcus* (3), *Photobacterium* (1), *Proteus* (1)*, Pseudomonas* (3), *Serratia* (2) and *Shewanella* (3) as shown in Table 2. Due to the phenotypic characteristic method is difficult and time-labor consuming, thus genotypic characterization by using 16S rRNA gene identification were implemented. From Table 3, data showed that most of bacteria (149) found in all squid samples, during storage at 4°C for 14 days, were identified as *Aeromonas* (35), *Shewanella* (31), *Pseudomonas* (29), *Enterobacter* (11), *Carnobacterium* (5), *Lactococcus* (5), *Macrococcus* (5), *Brochothrix* (3), *Acinetobacter* (3), *Hafnia* (3), *Citrobacter* (2), *Vibrio* (2), *Chryseobacterium* (2), *Buttiauxella* (2), *Klebsiella* (1) *Leuconostoc* (1), *Psychrobacter* (1), *Lactobacillus* (1), *Enterococcus* (1), *Exiguobacterium* (1), *Bacillus* (1) and *Photobacterium* (1).

Table 2. Phenotypic characteristics of selected strains isolated from squid samples during refrigerated storage.

Isolated medium	Strain	Gram	Shape	Pigmentation	H_2S production	Catalase	Oxidase	Arginine	Spoilage substrate			Identified as (Genus)
									Adenine	Cysteine	Methionine	
Aeromonas	AE1-2	-	Rod	-	-	-	-	+	-	-	-	*Aeromonas*
isolation media	AE4-4	-	Rod	-	-	-	-	+	-	-	-	*Aeromonas*
	AE8-1	-	Rod	-	-	-	-	+	-	-	-	*Aeromonas*
Iron agar	IR2-2	-	Rod	+ (red)	+	-	-	-	+	+	+	*Shewanella*
	IR4-2	-	Rod	+ (pink)	+	-	-	-	+	+	+	*Shewanella*
	IR4-5	-	Rod	+ (pink)	+	-	-	-	+	+	+	*Shewanella*
Plate count	PC2-3	-	Rod	-	-	+	-	-	-	-	-	*Proteus*
agar	PC4-2	-	Rod	-	-	+	+	-	+	-	-	*Photobacterium*
	PC8-2	-	Rod	+ (red)	-	+	-	-	-	-	-	*Serratia*
Pseudomonas	PS2-2	-	Rod	-	-	+	+	-	-	-	-	*Pseudomonas*
isolation media	PS4-2	-	Rod	-	-	+	+	-	-	-	-	*Pseudomonas*
	PS4-3	-	Rod	-	-	+	+	-	-	-	-	*Pseudomonas*
Violet red bile	VR4-2	-	Rod	-	-	+	-	-	-	-	-	*Enterobacter*
glucose agar	VR6-3	-	Rod	-	-	+	-	-	-	-	-	*Enterobacter*
	VR8-4	-	Rod	+ (red)	-	+	-	-	-	-	-	*Serratia*
KF streptococci	EC2-3	+	Cocci	-	-	-	-	-	-	-	-	*Enterococcus*
agar	EC4-5	+	Cocci	-	-	-	-	-	-	-	-	*Enterococcus*
	EC6-4	+	Cocci	-	-	-	-	-	-	-	-	*Enterococcus*

H_2S: hydrogen sulphide

Table 3. Genotypic identification of selected strains isolated from squid samples during refrigerated storage.

Isolating Media	Strain	BLAST (Highest similarity) species	% of highest similarity
Aeromonas isolation media	AE2-36	*Aeromonas allosaccharophila*	100
	AE0-13	*Aeromonas dhakensis*	100
	AE0-11	*Aeromonas ichthiosmia*	100
	*AE1-2	*Aeromonas media*	99.72
	AE0-31	*Aeromonas molluscorum*	99.86
	AE0-33	*Aeromonas molluscorum*	100
	AE2-32	*Aeromonas salmonicida* subsp. *masoucida*	100
	AE2-34	*Aeromonas salmonicida* subsp. *masoucida*	100
	AE2-37	*Aeromonas taiwanensis*	99.16
	AE2-21	*Aeromonas veronii*	100
	AE2-33	*Aeromonas veronii*	99.86
	AE1-31	*Citrobacter murliniae*	99.72
	AE0-12	*Photobacterium leiognathi*	99.58
	AE1-12	*Pseudomonas deceptionensis*	99.86
	AE10-11	*Pseudomonas lundensis*	99.72
	AE10-13	*Pseudomonas lundensis*	99.71
	*AE4-4	*Pseudomonas lundensis*	99.86
	AE14-12	*Pseudomonas lundensis*	99.86
	*AE8-1	*Pseudomonas lundensis*	99.86
	AE0-32	*Vibrio campbellii*	99.72
	AE1-11	*Vibrio owensii*	99.71
	C2	*Aeromonas ichthiosmia*	100
	C7	*Aeromonas ichthiosmia*	98.95
	C8	*Aeromonas ichthiosmia*	100
	C1	*Aeromonas sobria*	100
	C5	*Shewanella baltica*	99.03
	C6	*Shewanella baltica*	99.16

Table 3. (Cont.)

Isolating Media	Strain	BLAST (Highest similarity) species	% of highest similarity
KF streptococci agar	EC0-31	*Enterobacter faecalis*	100
	EC0-32	*Enterobacter faecalis*	100
	EC1-31	*Enterobacter faecalis*	100
	EC8-21	*Enterobacter faecalis*	99.86
	EC8-31	*Enterobacter faecalis*	99.86
	EC10-21	*Enterobacter faecalis*	99.73
	EC12-11	*Enterobacter faecalis*	99.73
	EC14-21	*Enterobacter faecalis*	99.86
	EC14-31	*Enterobacter faecalis*	99.86
MRS agar	L3	*Carnobacterium divergens*	99.63
	L5	*Lactobacillus sakei* subsp. *sakei*	100
	L1	*Lactococcus garvieae*	99.86
	L4	*Lactococcus garvieae*	99.86
	L9	*Lactococcus garvieae*	99.72
	L10	*Lactococcus garvieae*	99.58
	L2	*Lactococcus lactis* subsp. *lactis*	99.59
	L7	*Leuconostoc lactis*	100
Aeromonas agar	M11	*Acinetobacter schindleri*	97.1
	M6	*Aeromonas allosaccharophila*	100
	M9	*Aeromonas allosaccharophila*	99.72
	M7	*Aeromonas dhakensis*	99.84
	M17	*Aeromonas salmonicida* subsp. *salmonicida*	99.86
	M16	*Aeromonas veronii*	99.72
	M14	*Carnobacterium maltaromaticum*	100
	M1	*Enterococcus casseliflavus*	99.86
	M15	*Exiguobacterium indicum*	99.44
	M21	*Hafnia alvei*	99.86
	M8	*Macrococcus caseolyticus*	99.86
	M10	*Macrococcus caseolyticus*	99.72
	M13	*Macrococcus caseolyticus*	100
	M18	*Macrococcus caseolyticus*	99.72

Table 3. (Cont.)

Isolating Media	Strain	BLAST (Highest similarity) species	% of highest similarity
Aeromonas agar	M23	*Macrococcus caseolyticus*	100
	M12	*Pseudomonas fragi*	99.3
	M2	*Pseudomonas lundensis*	99.86
	M3	*Pseudomonas lundensis*	99.86
	PS2-21	*Aeromonas allosaccharophila*	100
	PS0-12	*Aeromonas hydrophila* subsp. *anaerogenes*	100
	PS1-21	*Aeromonas hydrophila* subsp. *anaerogenes*	100
	PS0-32	*Aeromonas ichthiosmia*	99.86
	PS2-11	*Aeromonas media*	99.16
	PS1-31	*Aeromonas molluscorum*	100
	PS0-33	*Aeromonas piscicola*	100
	PS0-35	*Aeromonas punctata* subsp. *caviae*	99.02
	PS4-21	*Aeromonas salmonicida* subsp. *pectinolytica*	100
	PS2-31	*Aeromonas veronii*	99.86
	PS10-33	*Carnobacterium divergens*	99.43
	PS12-21	*Carnobacterium divergens*	99.45
	PS10-31	*Pseudomonas gessardii*	100
	PS12-31	*Pseudomonas gessardii*	100
	PS12-32	*Pseudomonas gessardii*	100
	PS14-21	*Pseudomonas gessardii*	100
	PS0-31	*Shewanela baltica*	99.02
	PS0-34	*Shewanela baltica*	99.02
Pseudomonas isolation media	PS6-11	*Shewanella baltica*	99.02
	PS10-35	*Shewanella baltica*	99.58
	PS0-11	*Shewanella seohaensis*	99.72
Iron agar	S32	*Acinetobacter lwoffii*	97.78
	S04	*Acinetobacter schindleri*	97.02
	S18	*Aeromonas piscicola*	
	S07	*Aeromonas salmonica* subsp. *pectinolytica*	
	S02	*Bacillus cereus*	

Table 3. (Cont.)

Isolating Media	Strain	BLAST (Highest similarity) species	% of highest similarity
Pseudomonas isolation media	S03	*Brochothrix termosphacta*	
	S06	*Brochothrix termosphacta*	
	S08	*Brochothrix termosphacta*	
	S16	*Carnobacterium divergens*	
	S20	*Chryseobacterium antarcticum*	98.27
	S28	*Chryseobacterium chaponense*	97.75
	S11	*Pseudomonas deceptionensis*	
	S15	*Pseudomonas deceptionensis*	
	S17	*Pseudomonas fragi*	
	S30	*Pseudomonas fragi*	
	S31	*Pseudomonas fragi*	
	S10	*Pseudomonas lundensis*	98.73
	S23	*Pseudomonas lundensis*	
	S29	*Pseudomonas lundensis*	
	S22	*Pseudomonas psychrophila*	
	S25	*Psychrobacter lutiphocae*	98.02
	S01	*Shewanella baltica*	
	S05	*Shewanella baltica*	98.73
	S12	*Shewanella baltica*	
	S13	*Shewanella baltica*	
	S14	*Shewanella baltica*	
	S19	*Shewanella putrefaciens*	
	TS0-11	*Shewanela baltica*	99.02
	TS0-31	*Shewanela baltica*	99.02
	TS0-32	*Shewanela baltica*	99.02
	TS1-11	*Shewanela baltica*	99.3
	TS1-13	*Shewanela baltica*	99.02
	TS1-22	*Shewanella baltica*	99.3
	TS8-31	*Shewanella baltica*	99.02
	TS10-11	*Shewanella baltica*	98.73

Table 3. (Cont.)

Isolating Media	Strain	*BLAST (Highest similarity) species*	% of highest similarity
Pseudomonas isolation media	TS10-32	*Shewanella baltica*	99.02
	TS10-21	*Shewanella baltica*	99.3
	TS10-22	*Shewanella baltica*	99.02
	TS10-31	*Shewanella baltica*	99.3
	TS12-11	*Shewanella baltica*	99.44
	TS14-11	*Shewanella baltica*	99.02
	TS12-21	*Shewanella putrefaciens*	99.86
	TS1-12	*Shewanella seohaensis*	99.44
	TS1-21	*Shewanella seohaensis*	99.72
Violet red bile glucose agar	VR0-21	*Aeromonas hydrophila* subsp. *anaerogenes*	100
	VR0-32	*Aeromonas media*	99.02
	VR0-22	*Aeromonas punctata* subsp. *caviae*	99.02
	VR10-32	*Buttiauxella gaviniae*	99.38
	VR6-11	*Buttiauxella noackiae*	100
	VR0-31	*Citrobacter murliniae*	99.72
	VR0-36	*Enterobacter cancerogenus*	99
	VR0-35	*Enterobacter mori*	99.15
	VR6-31	*Hafnia alveri*	99.86
	VR12-11	*Hafnia alveri*	99.86
	VR0-34	*Klebsiella michiganensis*	99.72
	VR0-33	*Leclercia adecarboxylata*	99.86
	VR1-31	*Pseudomonas fragi*	99.3
	VR1-11	*Pseudomonas japonica*	98.89
	VR10-11	*Pseudomonas jessenii*	99
	VR6-21	*Pseudomonas lundensis*	99.86
	VR6-31	*Pseudomonas lundensis*	99.72
	VR6-32	*Pseudomonas lundensis*	99.72
	VR14-21	*Pseudomonas lundensis*	99.72
	VR10-31	*Serratia plymuthica*	97.92
	VR14-11	*Serratia quinivorans*	99.71
	VR0-11	*Shewanella seohaensis*	100

3. Study on the impact of selected isolate on quality changes of squid during refrigerated storage

Seventeen identified isolated were selected to study on the impact on quality changes of squid during storage in refrigerator (4°C). They were selected based on major population found in spoilage squid, after 2 days of refrigerated storage in previous section. Seventeen identified isolate were as follow

1) *Shewanella baltica*
2) *Shewanella seohaensis*
3) *Shewanella putrefaciens*
4) *Hafnia alveri*
5) *Pseudomonas lundensis*
6) *Pseudomonas fragi*
7) *Pseudomonas deceptionensis*
8) *Pseudomonas gessardii*
9) *Serratia quinivorans*
10) *Brochothrix termosphacta*
11) *Chryseobacterium antarcticum*
12) *Macrococcus caseolyticus*
13) *Lactococcus garvieae*
14) *Aeromonas salmonica*
15) *Aeromonas media*
16) *Aeromonas molluscorum*
17) *Aeromonas hydrophila* subsp. *anaerogenes*

Aliquot of inoculate (6 Log cfu/ml) was transferred to a surface of squid mantles which place of sterile plastic tray and kept in refrigerator for 14 days. From Figure 4, it was found that all bacterial inoculated in squid samples growth rapidly and reached 8 Log cfu/g in 2 days of storage. No marked difference in microbial counts were found between all samples during storage of 5 days excepting *Aeromonas hydrophila* which increased from 5.27 to 9.5 Log cfu/g in 5 days. The results indicated that storage at refrigerated temperature were the optimal condition for these spoilage bacteria. Thus *A. hydrophila* was selected for further study.

pH of all squid samples at different time are shown in Fiigure 4. The initial pH of whole squid samples were in range of 5.94-6.42. pH of squid samples increased continuously during storage for 2-5 days. The rate of increase were greater in sample with *A. hydrophila* within 3 days

of storage. The increase in pH is generally associated with the formation of volative bases (Ohashi *et al*., 1991).

Moreover, The increases in microbial count were in agreement with the increases in TVB, TMA and pH (Figure 4). The initial TVB contents of squid mantles were range of 3.36 – 5.42. TVB content of all squid samples increased gradually within 3 days of storage, excepting squid inoculated with *A. hydrophila* which increased gradually within 2 days.

TMA content of all squid samples increased markedly during 2 days of storage (Figure 4). In general, the resullts of TMA changes in squid were similar to those of TVB changes throughout the storage. TMA is produced by the reduction of trimethylamine oxide (TMAO) by TMAO reductase-producing organisms (Adam and Moss, 2000). TMA contents is an indicator of bacterial deterioration in marine products (Seibel and Walsh, 2002). TMA has been found to be related with a loss of freshness of marine fishery products (Alur *et al*., 1995; Gram and Huss, 1996). The higher TVB and TMA contents were observed in squid inoculated with *A. hydrophila* compare with those other identified isolates, suggesting that *A. hydrophila* was specific spoilage organism of squid.

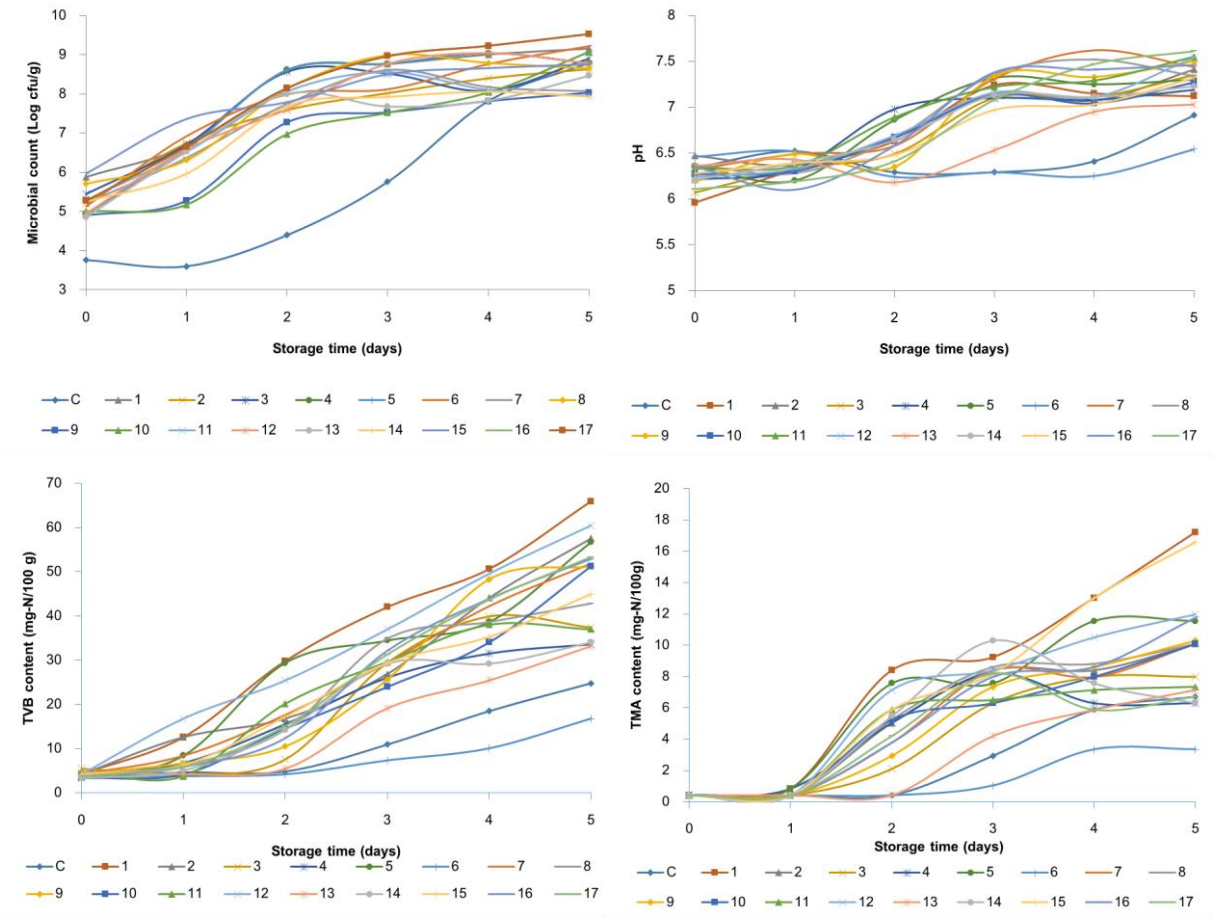

Figure 4. Microbial count, pH, TVB and TMA contents of squid mantle inoculated with selected isolated strains during refrigerated storage (4°C). Bars represent the standard deviation (n = 3).

4. Study on the effects of antimicrobial agents and packaging atmosphere on quality changes of squid during refrigerated storage

Effect of antimicrobial agents

Citric acid and potassium sorbate were used as antimicrobial in this study. Psychophilic bacteria and *Aeromonas* count of 1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning increased continuously during the storage up to 14 days (Figure 5). For all squid samples, the higher number of psychrophilic bacteria and *Aeromonas* were detected in sample without antimicrobial agent and it noticeable when the storage time was greater than 2 days. While samples treated with citric acid were lower in Psychophilic bacteria and *Aeromonas* count compare to potasium sorbate. For example, PBC of squid treated with citric acid increased from 3.71 to 7.04 Log cfu/g after 4 days of storage while those treated with potassium sorbater were from 3.91 to 7.54 Log cfu/g (Figure 5A). The results indicated that antimicrobial could retard the growth of microorganisms of squids, squids with evisceration and squid with evisceration and skinning and the shelf-life of all samples was longer than control.

Moreover, The increases in microbial count were in agreement with the increases in TVB, TMA and pH (Figure 6). The initial TVB contents of squids, squids with evisceration and squid with evisceration and skinning were 4 to 10 mg-N/100g. TVB content of all squid without antimicrobial agent increased gradually within the 4 days of storage while TVB content of antimicrobial treated increased gradually within 6 days.

TVB has been considered as usefull as a spoilage indicator (Yamanaka *et al*., 1987; Ohashi *et al*., 1991; Sungsri-in, 2010). At the same time of storage, especially during 6-14 days, the sample without any treatment (whole squid) had the higher TVB content, compared with those squids with evisceration and skinning. TMA content of squid without evisceration and skinning increased markedly during 4-14 days of storage (Figure 6). The higher TVB and TMA contents were observed in whole squid compare with those evisceration and skinning. Furthermore, skinning and evisceration of squid tended to lower the rate of increase in both TVB and TMA contents during storage.

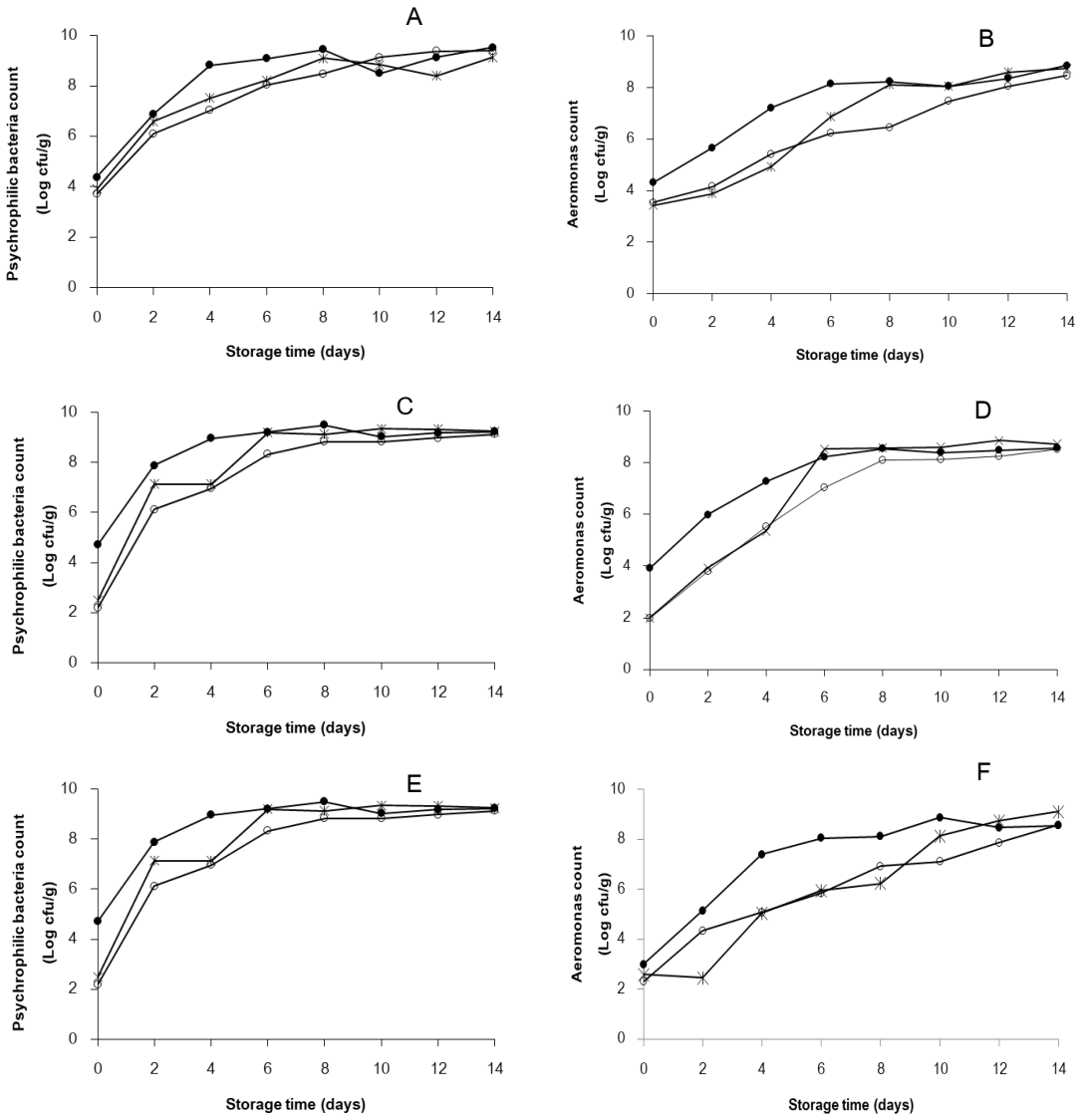

Figure 5. Psychrophilic bacteria and Aeromonas counts of whole squids (A and B), squids with evisceration (C and D) and squids with evisceration and skinning (E and F) treated with 3% NaCl solution (●) (control), 3% NaCl solution containing 0.2% citric acid (○) and 3% NaCl solution containing 0.45% potassium sorbate (*) during refrigerated storage (4°C). Bars represent the standard deviation (*n* = 3).

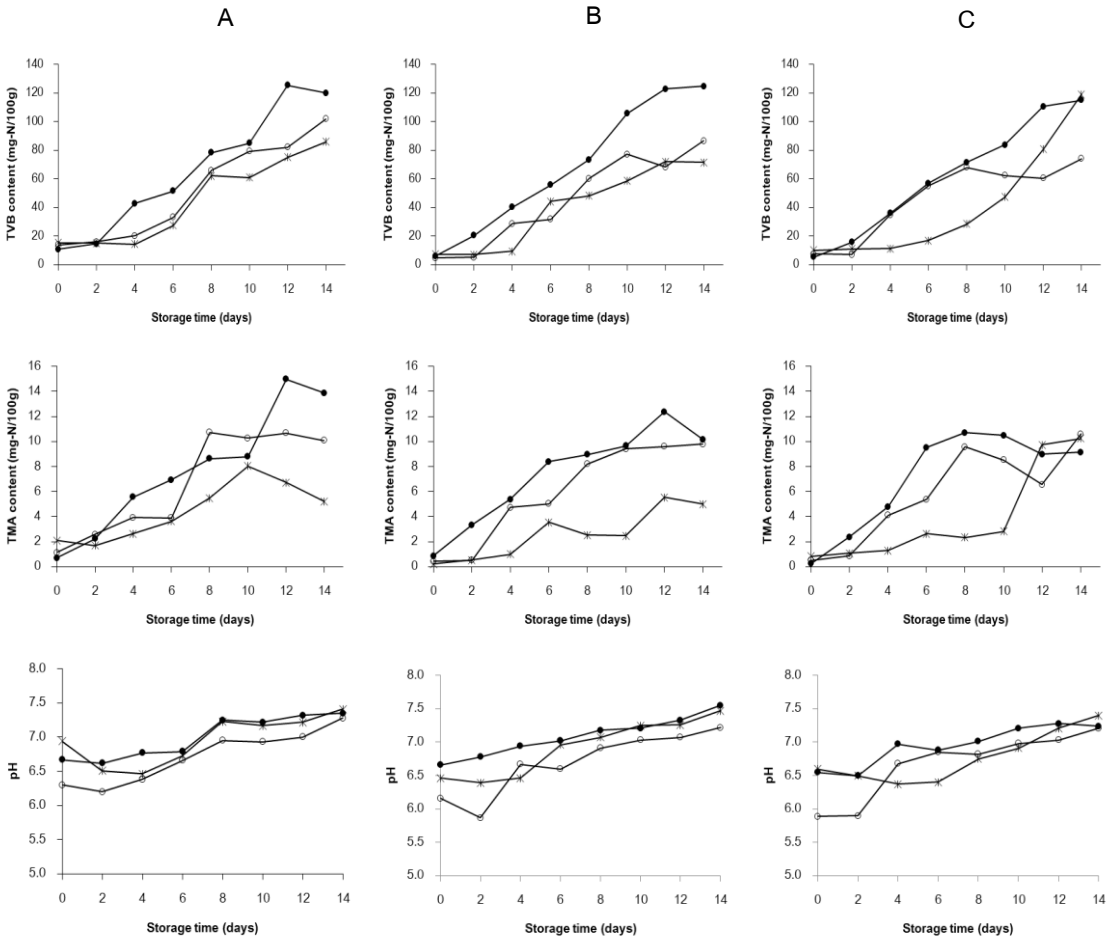

Figure 6. TVB, TMA contents and pH of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (D) treated with 3% NaCl solution (●) (control), 3% NaCl solution containing 0.2% citric acid (○) and 3% NaCl solution containing 0.45% potassium sorbate (*) during refrigerated storage (4°C). Bars represent the standard deviation (*n* = 3).

Moreover, the a* and b*-values of all samples increased up to 14 days of storage, except the a*-values of squids with evisceration and skinning increased. The a*-value of mantle from the squid without skinning for bothe whole squid and squid with evisceration increased continuously with increasing storage time, indicating the formation of pink color on squid mantle (Figure 7). the increase in a*-values was more pronounced in the samples after 4 days of storage. No changes in a*-values were observed in the mantle of deskinned squid throughot the storage. Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased.

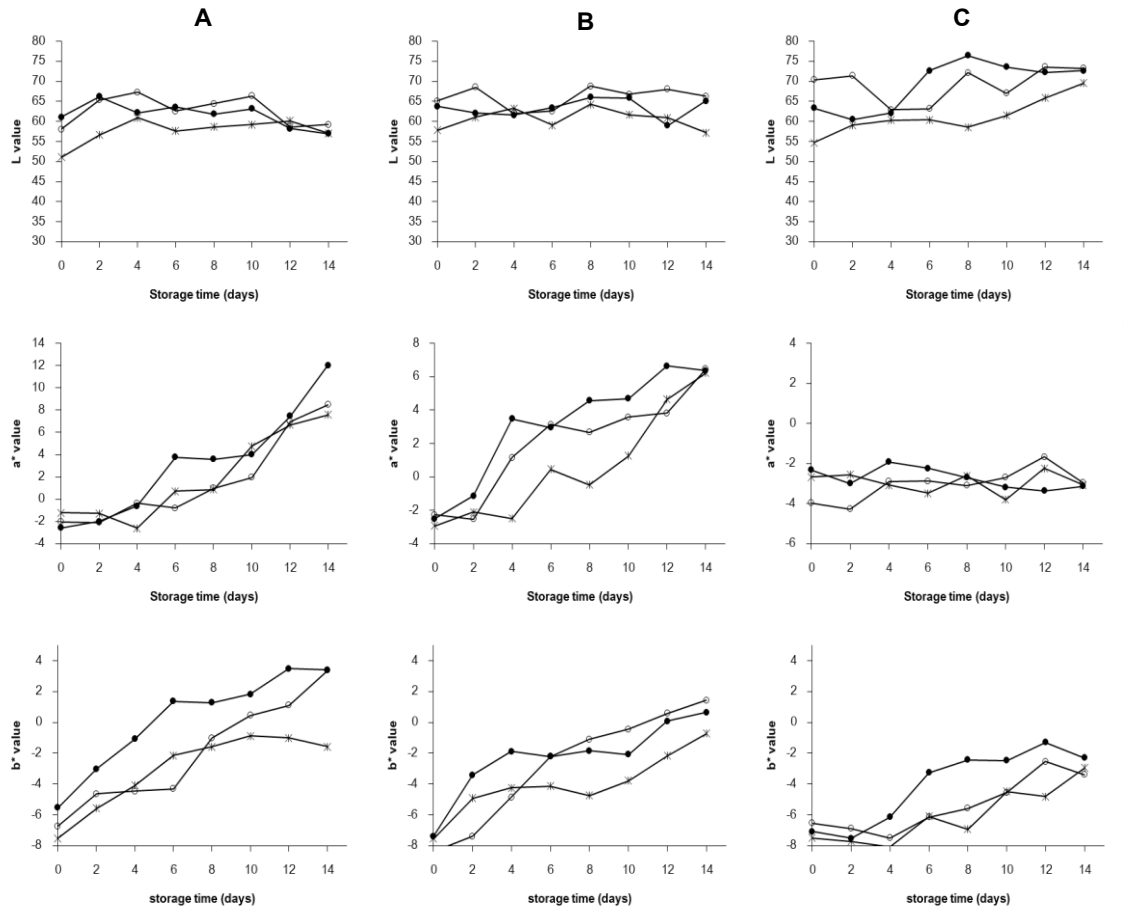

Figure 7. The L*, a* and b*-values of the mantle of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) treated with 3% NaCl solution (●) (control), 3% NaCl solution containing 0.2% citric acid (○) and 3% NaCl solution containing 0.45% potassium sorbate (*) during refrigerated storage (4°C). Bars represent the standard deviation (*n* = 3).

For b*-value representing the yellowness of mantle, all samples had the increased in b*-value up to 14 days. It was noted that the mantle of squid without skinning exhibited the higher increasses in b*-value, compared with the deskinned counterpart.

Effect of packaging atmospheres

Packaging atmospheres used in this study were of ambient atmosphere, vacuum and modified atmosphere packaging (60% CO_2, 30% N_2 and 10% O_2). Psychophilic bacteria and *Aeromonas* count of 1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning increased continuously during the storage up to 14 days (Figure 8). For all squid samples, the higher number of psychrophilic bacteria and *Aeromonas* were detected in sample packed with ambient atmosphere and it noticeable when the storage time was greater than 4 days. While samples packed in vacuum and MAP were lower in Psychophilic bacteria and *Aeromonas* count compare to ambient atmosphere. For example, PBC of whole squid packed in MAP increased from 3.82 to 6.54 Log cfu/g after 12 days of storage while those packed in vacuum were from 3.86 to 7.20 Log cfu/g after 10 days of storage (Figure 8A). The results indicated that packaging atmosphere could retard the growth of microorganisms of squids, squids with evisceration and squid with evisceration and skinning and the shelf-life of all samples was longer than control.

Moreover, The increases in microbial count were in agreement with the increases in TVB, TMA and pH (Figure 9). The initial TVB contents of squids, squids with evisceration and squid with evisceration and skinning were 9 to 20 mg-N/100g. TVB content of all squids with ambient atmosphere increased within the 2 days of storage while TVB content of squids packed in vacuum and MAP increased within 4 days. At the same time of storage, the sample packed in MAP had the lower TVB and TMA contents, compared with those squids packed in ambient atmosphere and vacuum packaging.

In addition, the a* and b*-values of all samples increased up to 14 days of storage, except the a*-values of squids with evisceration and skinning increased. The a*-value of mantle from the squid without skinning for bothe whole squid and squid with evisceration increased continuously with increasing storage time, indicating the formation of pink color on squid mantle (Figure 10). the increase in a*-values was more pronounced in the samples after 6 days of storage. No changes in a*-values were observed in the mantle of deskinned squid throughot the storage. Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased.

For b*-value representing the yellowness of mantle, all samples had the increased in b*-value up to 14 days. It was noted that the mantle of squid without skinning exhibited the higher increasses in b*-value, compared with the deskinned counterpart.

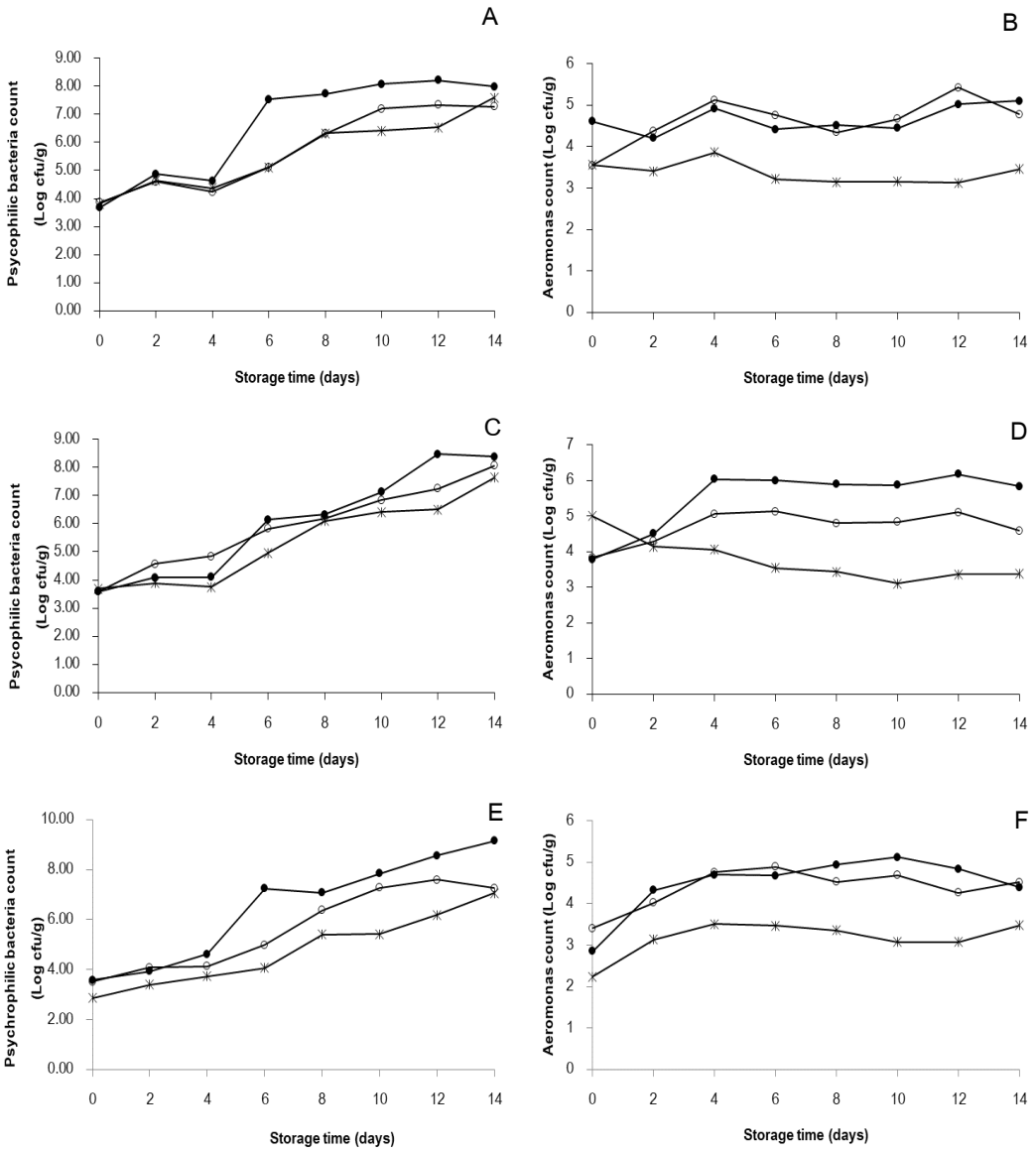

Figure 8. Psychrophilic bacteria and Aeromonas counts of whole squids (A and B), squids with evisceration (C and D) and squids with evisceration and skinning (E and F) kept in air (●) (control), vacuum (○) and MAP (60% CO_2, 30% N_2 and 10% O_2) (*) conditional pagkaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

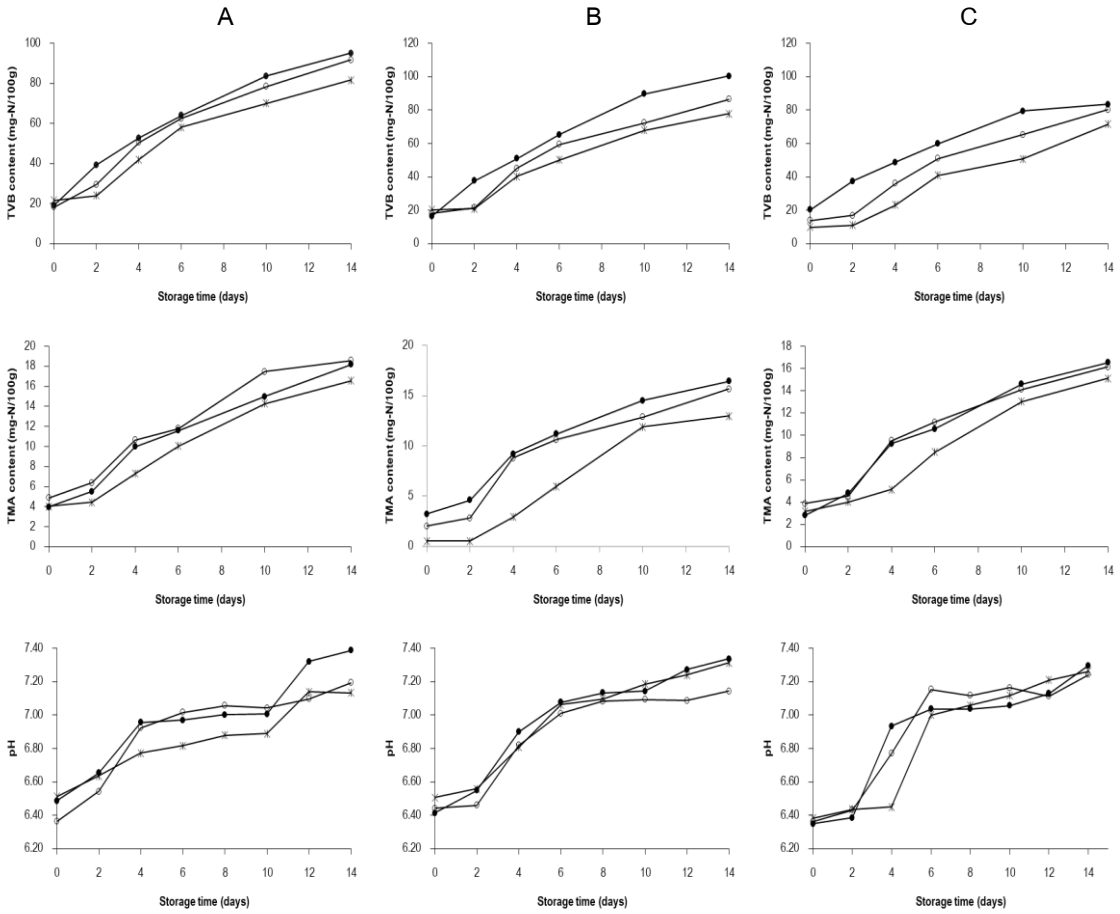

Figure 9. TVB, TMA contents and pH of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) kept in air (●) (control), vacuum (○) and MAP (60% CO_2, 30% N_2 and 10% O_2) (*) conditional pagkaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

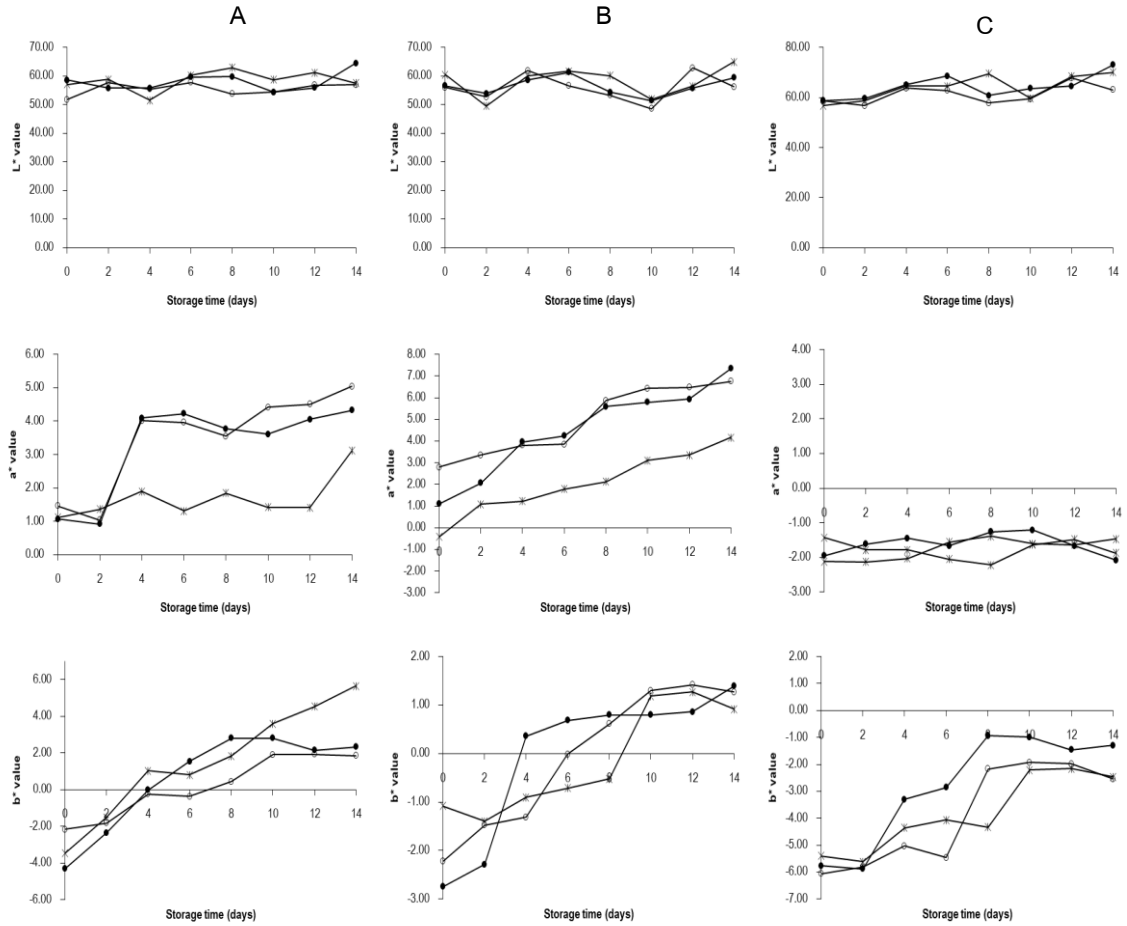

Figure 10. The L*, a* and b*-values of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) kept in air (●) (control), vacuum (○) and MAP (60% CO_2, 30% N_2 and 10% O_2) (*) conditional pagkaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

Effect of combination methods

Microbial count of 1) whole squids, 2) squids with evisceration and 3) squids with evisceration and skinning increased continuously during the storage up to 14 days (Figure 11). Squid samples packed in MAP with citric acid treated were lower in psychophilic bacteria and *Aeromonas* count compared to those packed in MAP without citric acid treated and it noticeable when the storage time was greater than 6 days. While samples packed in MAP with citric acid treated were lower in Psychophilic bacteria and *Aeromonas* count, for example, PBC of whole squid packed in MAP with citric acid treated increased from 3.9 to 7.43 Log cfu/g after 10 days of storage while those squid with evisceration and skinning were from 4.6 to 7.7 Log cfu/g after 10 days of storage (Figure 11). The results indicated that MAP combined with citric acid treated could retard the growth of microorganisms of squids, squids with evisceration and squid with evisceration and skinning and the shelf-life of all samples was longer than control.

Moreover, The increases in microbial count were in agreement with the increases in TVB, TMA and pH (Figure 12). The initial TVB contents of squids, squids with evisceration and squid with evisceration and skinning were 9 to 20 mg-N/100g. TVB content of all squids packed in MAP without citric acid increased within the 2 days of storage while TVB content of squids packed in MAP with citric acid treat increased within 6 days. At the same time of storage, the sample (whole squid, squid with evisceration and squid with evisceration and skinning) packed in MAP with citric acid treated had the lower TVB and TMA contents, compared with those squids packed in MAP without citric acid treated.

In addition, the a* and b*-values of all samples increased up to 14 days of storage, except the a*-values of squids with evisceration and skinning increased. The a*-value of mantle from the squid without skinning for bothe whole squid and squid with evisceration increased continuously with increasing storage time, indicating the formation of pink color on squid mantle (Figure 13). the increase in a*-values was more pronounced in the samples after 6 days of storage. No changes in a*-values were observed in the mantle of deskinned squid throughot the storage. Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased.

For b*-value representing the yellowness of mantle, all samples had the increased in b*-value up to 14 days. It was noted that the mantle of squid without skinning exhibited the higher increasses in b*-value, compared with the deskinned counterpart.

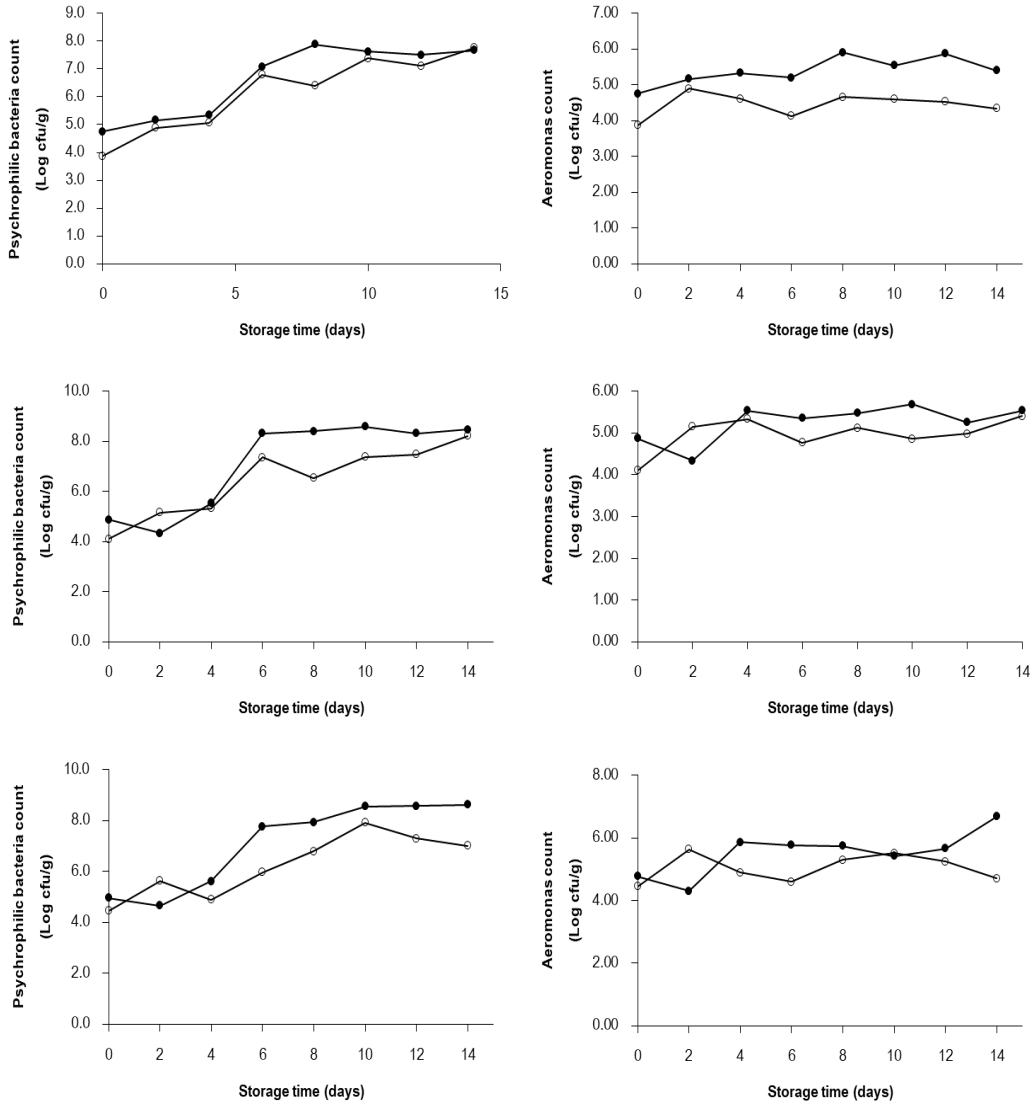

Figure 11. Psychrophilic bacteria and *Aeromonas* counts of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) treated with 3% NaCl solution (●) (control) and treated with 3% NaCl solution containing 0.2% citric acid (○) under MAP (60% CO_2, 30% N_2 and 10% O_2) packaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

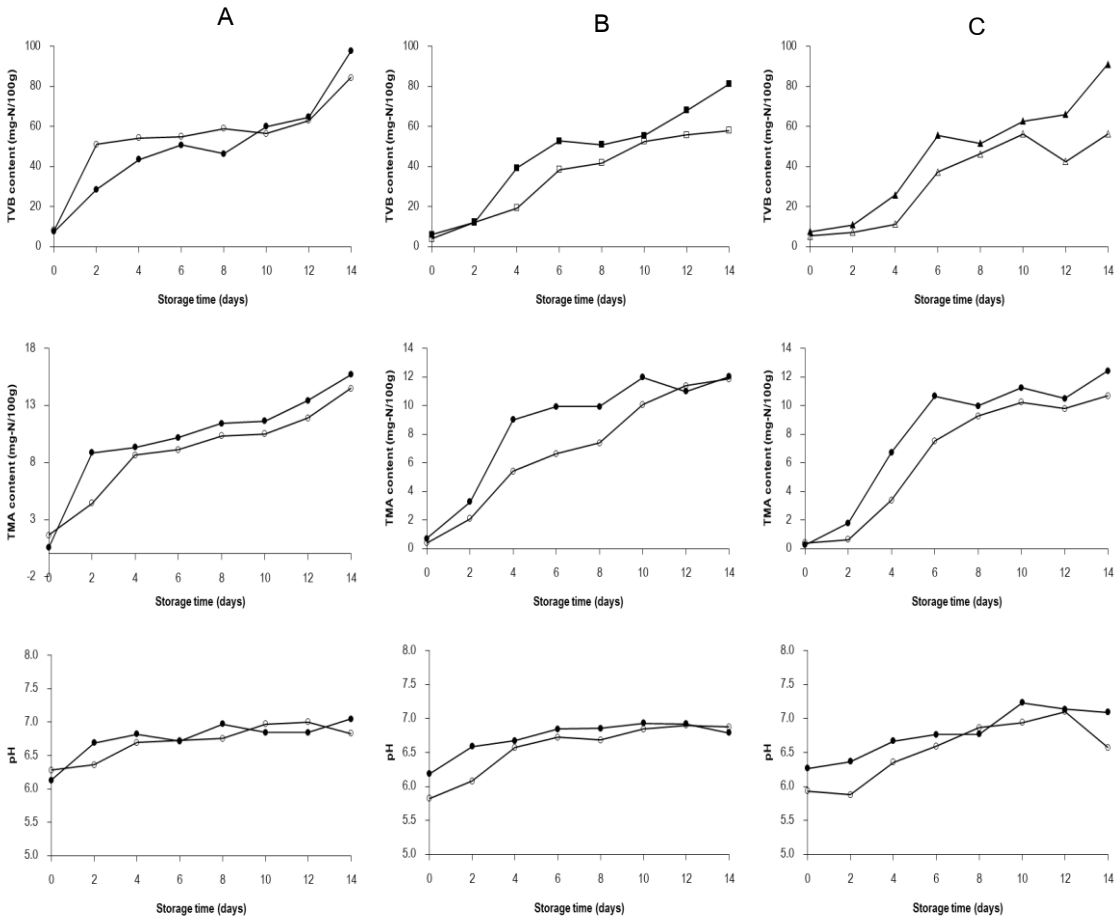

Figure 12. TVB, TMA contents and pH of of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) treated with 3% NaCl solution (●) (control) and treated with 3% NaCl solution containing 0.2% citric acid (○) under MAP (60% CO_2, 30% N_2 and 10% O_2) packaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

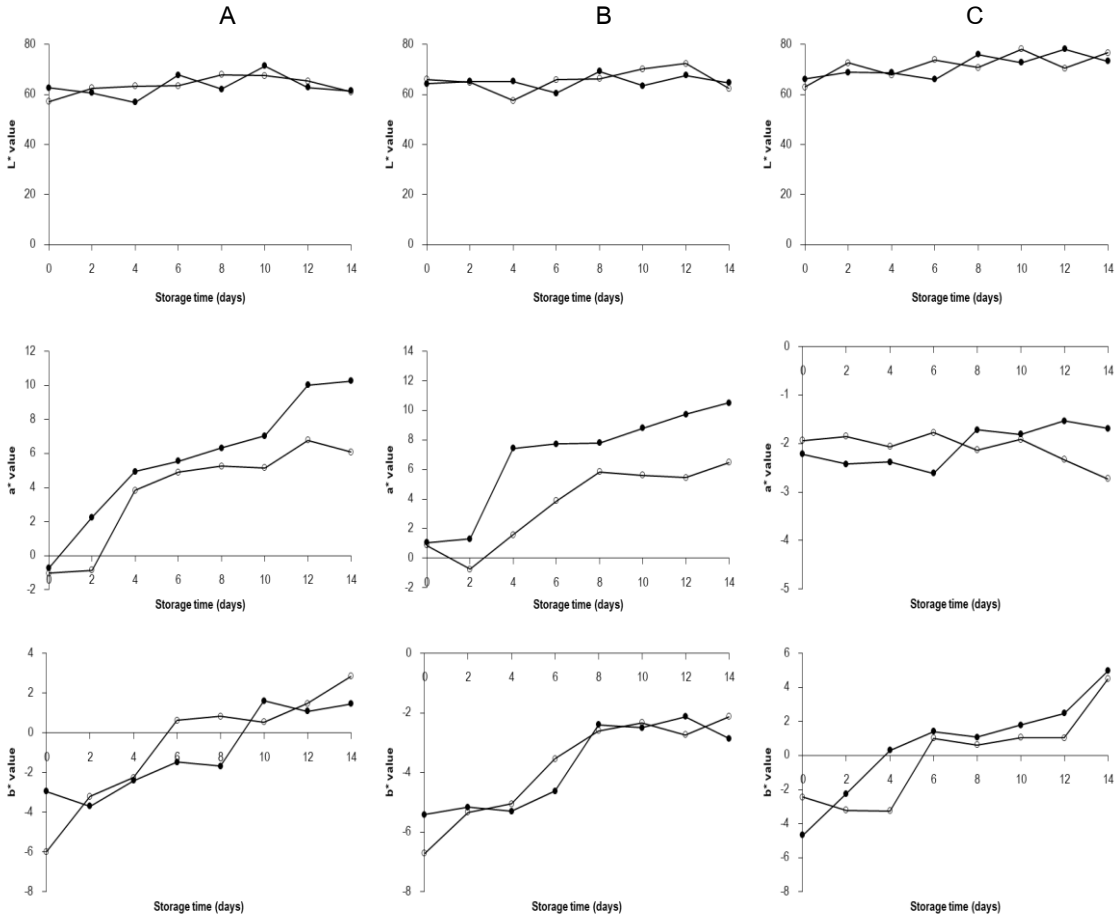

Figure 13. The L*, a* and b*-values of of whole squids (A), squids with evisceration (B) and squids with evisceration and skinning (C) treated with 3% NaCl solution (●) (control) and treated with 3% NaCl solution containing 0.2% citric acid (○) under MAP (60% CO_2, 30% N_2 and 10% O_2) packaging during refrigerated storage (4°C). Polyamide/poethylel (PA/PE) 20/70 pouches with 90 μm in thickness bags were used. Bars represent the standard deviation (*n* = 3).

Thus, the combination of packaging with antimicrobial treated could retard the spoilage of squids (whole squid, squid with evisceration and squid with evisceration and skinning) for longer than 6 days.

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