การคัดเลือกแลกติกแอซิดแบคทีเรียที่สร้างแบคเทอริโอซินจากแหนมเพื่อยับยั้งแบคทีเรียก่อโรค

นางสาวกัญชนิกา รุ่งเริงสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

SELECTION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM NHAM FOR INHIBITION OF PATHOGENIC BACTERIA

Miss Kanchaniga Rungreangsuk

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology

Department of Microbiology
Faculty of Science
Chulalongkorn University

Academic Year 2008

Thesis Title	SELECTION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM NHAM FOR INHIBITION OF PATHOGENIC BACTERIA
By	Miss Kanchaniga Rungreangsuk
Field of Study	Industrial Microbiology
Thesis Advisor	Associate Professor Sirirat Rengpipat, Ph.D.

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

S. Hannongbua ..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

Prakitsin Sihanonth ..... Chairman

(Associate Professor Prakitsin Sihanonth, Ph.D.)

Sirirat Rengpipat ..... Thesis Advisor

(Associate Professor Sirirat Rengpipat, Ph.D.)

Jiraporn Thaniyavarn ..... Examiner

(Associate Professor Jiraporn Thaniyavarn)

Sukjai Choojun ..... External Examiner

(Associate Professor Sukjai Choojun)

กัญชนิกา รุ่งเรืองสุข : การคัดเลือกแลกติกแอซิดแบคทีเรียที่สร้างแบคเทอริโอซินจากแหนมเพื่อยับยั้งแบคทีเรียก่อโรค. (SELECTION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM NHAM FOR INHIBITION OF PATHOGENIC BACTERIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ศิริรัตน์ เร่งพิพัฒน์, 196 หน้า.

คัดเลือกแลกติกแอซิดแบคทีเรียที่สามารถสร้างแบคเทอริโอซินจาก 107 ไอโซเลตที่แยกได้จากแหนม 20 ตัวอย่าง ที่ผลิตจากบริษัทสุทธิลักษณ์ อินโนฟู้ด จำกัด โดยวิธี agar spotted test และ agar well diffusion. มีเพียง 34 ไอโซเลต ที่สร้างแบคเทอริโอซินยับยั้ง *Staphylococcus aureus*. ไอโซเลต NSL13-2, NSL1-4 และ NSL5-2 สามารถยับยั้ง *S. aureus* ได้มากที่สุด เมื่อนำมาศึกษาลักษณะสัณฐานวิทยา ลักษณะทางชีวเคมีโดยใช้ API 50 CHL (BioMérieux, France) และการวิเคราะห์ลำดับนิวคลีโอไทด์ของยีน 16S rDNA พบว่าทั้ง 3 ไอโซเลต คือ *Pediococcus pentosaceus*, *Weissella cibaria* และ *Enterococcus gilvus* ตามลำดับ. การยับยั้งแบคทีเรียก่อโรค 10^4 CFUml^{-1} พบว่ามีประสิทธิภาพสูงสุดเมื่อเพาะเลี้ยงร่วมกับ *P. pentosaceus* 10^6 CFUml^{-1} ในอาหารเลี้ยงเชื้อเหลว MRS โดย *S. aureus*, *Listeria monocytogenes* และ *Salmonella* Typhimurium ถูกยับยั้งสมบูรณ์หลังจากการบ่ม 12 ชั่วโมง ในขณะที่ *Bacillus cereus* และ *Escherichia coli* ถูกยับยั้งสมบูรณ์หลังจากการบ่ม 8 และ 16 ชั่วโมง ตามลำดับ. ศึกษาอิทธิพลของภาวะแวดล้อมที่มีผลต่อการเจริญ และการผลิตแบคเทอริโอซินของ *P. pentosaceus* พบว่า *P. pentosaceus* เจริญสูงสุดเมื่อเพาะเลี้ยงในอาหารเลี้ยงเชื้อเหลว MRS ที่ค่าความเป็นกรด-ด่าง 8.0 และบ่มที่ 30 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง. แบคเทอริโอซินผลิตสูงสุดเมื่อ *P. pentosaceus* เพาะเลี้ยงในอาหารเลี้ยงเชื้อเหลว MRS ที่เติม NaCl 3% (น้ำหนักต่อปริมาตร) ช่วงพิสัยของอุณหภูมิ 25 -37 องศาเซลเซียส ค่าความเป็นกรด-ด่าง 6.0-9.5 และเวลาการบ่มที่ 12 หรือ 24 ชั่วโมง เป็นภาวะที่เหมาะสมต่อการผลิตแบคเทอริโอซิน. เมื่อนำ *P. pentosaceus* มาประยุกต์ใช้เป็นหัวเชื้อสำหรับการเตรียมแหนมเพื่อศึกษาประสิทธิภาพในการยับยั้งแบคทีเรียก่อโรคในตัวอย่างอาหาร จากผลการทดลองพบว่า *S. aureus*, *B. cereus* และ *S.* Typhimurium ถูกยับยั้งสมบูรณ์ในแหนมที่เติมทั้ง *P. pentosaceus* 10^6 CFUg^{-1} และ แบคทีเรียก่อโรค 10^4 CFUg^{-1} หลังจากระยะเวลาการหมักที่ 120, 24 และ 120 ชั่วโมง ตามลำดับ ในขณะที่ *L. monocytogenes* และ *E. coli* พบว่ามีปริมาณลดลงเหลือเพียง 2.26 และ 3.23 log CFU ณ วันสุดท้ายของการหมัก ดังนั้นสามารถนำ *P. pentosaceus* มาใช้เป็นหัวเชื้อในการเตรียมแหนมเพื่อป้องกันอันตรายที่เกิดจากแบคทีเรียก่อโรคที่ปนเปื้อนจากวัตถุดิบ และระหว่างกระบวนการผลิตแหนมได้

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต กัญชนิกา รุ่งเรืองสุข

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก ศิริรัตน์ เร่งพิพัฒน์

ปีการศึกษา 2551

4972213123 : MAJOR INDUSTRIAL MICROBIOLOGY

KEYWORDS : LACTIC ACID BACTERIA / BACTERIOCIN / NHAM

KANCHANIGA RUNGREANGSUK : SELECTION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM NHAM FOR INHIBITION OF PATHOGENIC BACTERIA. THESIS ADVISOR : ASSOC. PROF. SIRIRAT RENGPIPAT, Ph.D., 196 pp.

A total of 107 lactic acid bacteria (LAB) isolated from 20 samples of Thai sour pork (nham), were collected from Suddhiluck Innofood Co.,Ltd. Bacteriocin-producing LAB were screened using an agar spotted test and agar well diffusion method. Among those LAB, only 34 isolates could inhibit *Staphylococcus aureus*. The most inhibition on *S. aureus* of these isolates namely NSL13-2, NSL1-4 and NSL5-2 were selected and identified based on morphology and biochemical characteristics using API 50 CHL (BioMérieux, France) and 16S rDNA analysis. NSL13-2, NSL1-4 and NSL5-2 were identified as *Pediococcus pentosaceus*, *Weissella cibaria* and *Enterococcus gilvus*, respectively. Growth inhibition of pathogenic bacteria was investigated by co-culturing with selected LAB in MRS broth. The highest efficiency of inhibitory effect observed when 10^4 $CFUml^{-1}$of pathogenic bacteria were co-cultured with 10^6 $CFUml^{-1}$ of *P. pentosaceus*. Complete growth inhibition of *S. aureus*, *Listeria monocytogenes* and *Salmonella* Typhimurium were detected after 12 hours of incubation, while *Bacillus cereus* and *Escherichia coli* were observed after 8 and 16 hours, respectively. Maximal growth of *P. pentosaceus* was obtained by culturing in MRS broth pH 8.0 and incubated at 30°C for 24 hours. Whereas, the highest bacteriocin activity was obtained by culturing in MRS broth adding with 3% (w/v) NaCl. Temperature, pH and incubation time that more favourable for bacteriocin production were range from 25 to 37°C, pH 6.0-9.5 and 12 or 24 hours. The application of 10^6 $CFUg^{-1}$ of *P. pentosaceus* as starter culture for nham preparation was performed for study inhibitory efficiency in food model. No detection of *S. aureus*, *B. cereus* and *S.* Typhimurium in nham inoculated both of pathogenic bacteria and *P. pentosaceus* (NSP) after 120, 24 and 120 hours of fermentation time, respectively. Whereas, decrease in number of *L. monocytogenes* and *E. coli* were observed. The number of *L. monocytogenes* and *E. coli* in NSP decreased to 2.26 and 3.23 log CFU at the end of fermentation, respectively. Therefore, *P. pentosaceus* could be used as starter culture in nham preparation in order to prevent the harmless of pathogenic bacteria.

Department : Microbiology Student's Signature Kanchaniga Rungreangsuk

Field of Study : Industrial Microbiology Advisor's Signature Sirirat Rengpipat

Academic Year : 2008

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Associate Professor Dr. Sirirat Rengpipat, for her kind guidance, assistance and grateful encouragement throughout the course of my research. In addition, I would like to express my thanks to thesis committee, Associate Professor Dr. Prakitsin Sihanonth, and Associate Professor Jiraporn Thaniyavarn for their comments and valuable suggestions in this research.

Gratefully thanks to Associate Professor Sukjai Choojun of Applied Biology Department, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang for her kind acting as external examiner, comments and valuable suggestions in this research.

I would like to thank for Suddhiluck Innofood Co.,Ltd for their kind providing of nham samples throughout of my research.

I would like to thank for CU Graduate School Thesis Grant of Chulalongkorn University for all support in this research.

Furthermore, I would like to thank all members and staff of Microbiology Department for advice, kind assistance and support of equipments throughout my work.

Moreover, I would like to express my thanks to my special friend for helping, encouragement, giving me good advices and not leaving me alone when I feel depression or have problems.

Special thanks for all members of laboratory room 408, Ms. Kankiya Chanitnun and Ms. Teeranun Suwanumphai for encouragement, nice friendships and giving me a good time at Chulalongkorn University. Also, I would like to thank my friends in Saint Joseph Bangna School and King Mongkut's Institute of Technology Ladkrabang for giving me encouragement and cheerfulness.

Finally, I would like to express my thanks to my family especially my lovely parents for their warmest love, care, encouragement, understanding, support and attention throughout my life.

CONTENTS

page

page

page

page

LIST OF TABLES

LIST OF FIGURES

FIGURE page

FIGURE page

CHAPTER I

INTRODUCTION

Nham is a Thai traditional sour pork, which is popularly consumed throughout all part of Thailand especially in Northeastern part. Nham is prepared from ground pork, pork rinds, garlic, cooked rice, salt, chili, sugar, pepper, sodium nitrite (Valyasevi, R. and Rolle, R.S., 2002) The uniqueness in texture, flavor, color and taste of nham are obtained from the spontaneous fermentation by indigenous microorganisms flora, which are normally found in raw materials (Visessanguan et al., 2006). In general, nham is produced in Thailand primarily by small-scale and cottage-level processor that the control of sanitation may improper for the prevention of contaminated foodborne or pathogenic microorganisms (Valyasevi, R. and Rolle, R.S., 2002) because the lack of scientific and technological knowledge are applied during fermentation processes. Moreover, nham is normally consumed without cooking, so the contaminated products can cause foodborne illnesses for the consumers and food safety problems.

Staphylococcus aureus, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli, Bacillus cereus* and other *Bacillus* species were considered as potential hazards and involved in foodborne outbreaks associated with the consumption of pork and poultry meat (Mataragas et al., 2008). The contamination of pathogenic bacteria is often found in raw meat result of human handling, meat handling surfaces and dressing of carcasses (Ayres, 1995). The presence of those pathogenic bacteria in meat products is a particular food safety concern. In spite of stability of product quality and ambiguous product safety, nham production has been developed by using a starter culture technology (Visessanguan et al., 2006).

Lactic acid bacteria (LAB) are more attractive for using as a starter culture because LAB are considered as GRAS (generally recognized as safe) organisms that are safe to consume and have long history of use in fermentation to preserve the nutritive qualities of food (Bredholt et al., 2001). Moreover, LAB have ability to produce various antimicrobial substances including lactic acid, hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl and bacteriocins (Daeschel et al., 1991). Bacteriocins are ribosomally synthesized antimicrobial peptides which have a bactericidal or bacteriostatic effect on other or closely related species (Garneau et al., 2002). Mode of action of bacteriocin is considered to act at cytoplasmic membrane. The pore formation and releasing of cytoplasmic materials was occurred leading to the death of sensitive cells (Montville et al., 1995).

Due to demanding of natural product of consumers were increased. Therefore, the use of bacteriocin-producing LAB has been attracted in food industry by using as protective culture. Thus, the objective of this research was to isolate and screen for bacteriocin-producing LAB from nham against certain pathogenic bacteria. The highest potential strain will be selected as starter culture for preparing nham, a model for this study.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 NHAM

Fermentation not only offers means of food preservation but also adapts in preparation of a variety of food products. Indigenous fermented foods were developed through traditional or village-art methodologies which were preserved in order to maintain the uniqueness and characteristic of these foods. Many products are produced by this process for example dairy products such as cheese, yoghurt and kefir; vegetable products such as kimchi, mixed pickles and sauerkraut; and meat products such as nham, som-fug and plaa-som. Among fermented meat products of Thailand, nham or a fermented pork sausage has achieved much popularity and acceptance due to its own uniqueness in texture, flavor, color and taste (Visessanguan et al., 2006).

Nham, a Thai traditional fermented pork sausage, is popularly consumed throughout all part of Thailand especially in Northeastern part. Nham is mainly prepared from ground pork, pork rinds, garlic, cooked rice, salt, chili, sugar, pepper, sodium nitrite (Valyasevi and Rolle, 2002). The uniqueness in texture, flavor, color and taste of nham are obtained from the spontaneous fermentation by indigenous microorganisms flora, which are normally found in raw materials (Visessanguan et al., 2006). Nham is generally consumed with chili pepper and ginger without further cooking.

2.2 Production of NHAM

Nham is prepared by combining a mixture of ground rice and garlic with dry ingredients including ground black pepper, salt, sugar and sodium nitrite. The mixture of ingredients is thoroughly mixed with lean meat which trimmed of all visible fat and connective tissue and boiled sliced pork rinds, and tightly packaged in banana leaves

or plastic sheets. The fermentation process of nham is spontaneous by predominant microorganisms flora in raw material such as Lactobacilli (*Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus sake*) and Pediococci (*Pediococcus acidilactici* and *Pediococcus pentosaceus*) (Tanasupawat and Komagata, 1995). Nham generally fermented at ambient temperature of 30°C for 3–4-day in order to achieve a final pH of 4.5. The pH drop is caused by producing organic acids from carbohydrate fermentation. Acid production contributes to both quality and safety in nham production, in that it imparts the sour taste, typical fermented aroma, firmness of texture and can prevent the growth of acid sensitive pathogens.

Figure 2.1 The production of nham by small-scale and cottage-level processor.
Available from: http://www.becnews.com/backissue/k_knowthai/knowthai220447.html [2008, December 28]

In general, nham is produced in Thailand primarily by small-scale and cottage-level processor (Figure 2.1) that the scientific and technological knowledge during fermentation processes are lacked and improper for the prevention of contaminated foodborne or pathogenic microorganisms. So, the quality and consistency of product cannot be controlled (Valyasevi and Rolle, 2002). Moreover, nham is produced from fresh meat which can provide an excellent environment for the growth of pathogenic and spoilage microorganisms and is normally consumed without cooking, so

proper acid production is only one factor for determining the quality and safety of nham. In nham with pH higher than 4.6, the contamination of pathogenic bacteria such as *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* was found, however; the severe of contamination depend on the initial number of contaminated microorganisms. So, the contaminated products can cause foodborne illnesses for the consumers and food safety problems. The development of controlled fermentation processes will become necessary in order to provide safe products with high quality for the consumers. To achieve for this purpose, the starter culture technology has been applied for the fermentation of meat products. The mainly bacteria that commonly found in fermented meat products and interested in the role of biopreservation is lactic acid bacteria (LAB) (Vermeiren et al., 2006).

The first starter culture formula for nham fermentation was developed by Wiriyacharee et al. (1990). The formula consisted of Lactobacilli, Pediococci and Micrococci along with fixed amounts of various ingredients that have been successfully and exclusively applied in the industrial production of nham. Valyasevi et al. (2001), who further developed the starter culture formula to improve and control of microbial processes and to maintain product diversity. They selected *Lactobacillus curvatus*, acid-producing bacteria commonly found in fermented meat products that have been presented to be one of the effective strains as nham starter culture.

2.3 Lactic acid bacteria (LAB)

2.3.1 Classification of Lactic acid bacteria

The general description of lactic acid bacteria (LAB) are a group of Gram-positive bacteria, non-sporing, cocci or rod shaped, catalase negative, microaerophile, fastidious, acid tolerant, which produce lactic acid as the major end product during the carbohydrate fermentation. The classification of LAB into different genera is based on morphology, mode of glucose fermentation, configuration of lactic acid produced,

growth at different temperature, ability to grow at high salt concentration, and acid or alkaline tolerance (Axelsson, 1998). Recent taxonomy of lactic acid bacteria comprises 12 genera following: *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, *Vagococcus*, *Tetragenococcus*, *Aerococcus*, *Oenococcus*, *Weissella* and *Carnobacterium*.

2.3.1.1 *Lactobacillus*

The genus *Lactobacillus* is rod-shaped LAB, which is the largest genus included in LAB. It is very heterogeneous because It is reflected by the range of mol% G+C of the DNA of species in the genus. This range is 32-53 %mol G+C, so LAB in this genus has a large variety of phenotypic, biochemical, and physiological properties (Schleifer and Stackebrandt, 1983). The Lactobacilli are strictly fermentative and have complex nutritional requirements. Moreover, Lactobacilli are widespread in many different habitats (table 2.1).

Table 2.1 Habitats of the genus *Lactobacillus*

Humans	Oral cavity, intestinal tract and vagina
Other habitats	Plants and plant materials Soil, water, sewage and manure Food fermentation (milk, meat, and vegetable) Cereal products and silage
Food spoilage	Beer and fermented beverages Fruit and grain mashes Marinated fish, meat and meat products Milk and sugar processing

Source: Holzapfel and Stiles (1997)

The classical ways for the distinction of Lactobacilli species based on the carbohydrate fermentation patterns, which can divide into 3 groups from the reviews by Hammes et al. (1991), Pot et al. (1994) and Vandamme et al. (1996).

Group 1 Obligately homofermentative Lactobacilli, which ferment hexose to lactic acid as the end product via the Embden–Meyerhof–Parnas pathway. Pentose and gluconate cannot be fermented by group 1 Lactobacilli because they are unable to produce phosphoketolase enzyme but they are only able to produce fructose-1, 6-bisphosphate-aldolase enzyme. The important food associated species in this group include *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus farciminis* and *Lactobacillus kefiranofaciens*.

Group 2 Facultatively heterofermentative Lactobacilli, which ferment hexose to lactic acid as the end product via the Embden–Meyerhof–Parnas pathway. Group 2 Lactobacilli can ferment both of pentose and gluconate because they are able to produce both of phosphoketolase and aldolase enzymes. The pentose fermentation produces lactic and acetic acids. In addition, the gluconate fermentation may produce gas. The important food associated species in this group include *Lactobacillus casei* and *Lactobacillus plantarum*.

Group 3 Obligately heterofermentative Lactobacilli, which ferment both of hexose and pentose to lactic acid, acetic acid, ethanol and carbon dioxide via the phosphogluconate pathway. The most important heterofermentative Lactobacilli associated with food fermentations are *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus kefir* and *Lactobacillus reuteri*.

2.3.1.2 *Streptococcus*

The genus *Streptococcus* is chain-forming and cocci or ovoid-shaped, 0.8-1.2 micrometer in diameter, homofermentative, growth at 20-42°C and have complex nutritional requirements. The range of G+C content is 32-53 %mol G+C (Hardie and

Whiley, 1995). This genus comprised a wide range of organisms including the highly pathogenic bacteria such as *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Streptococcus agalactiae*. In addition, *Streptococcus cremoris*, *Streptococcus lactis* and *Streptococcus thermophilus* are economically important for using as starter culture bacteria in many kinds of food, especially *S. thermophilus* is an important starter organism for yoghurt and cheese manufacture (Jones, 1978).

2.3.1.3 *Lactococcus*

The shaped of genus *Lactococcus* is difficult to interpret because the cells are sometimes elongated in the plane of chain formation. Normally, *Lactococcus* are cocci or ovoid shaped, 0.5-1.0 micrometer in diameter, produce only L(+)-lactic acid from glucose fermentation and growth at 10°C but not at 45°C. The range of G+C content is 34-43 %mol G+C. This genus comprises 5 species such as *Lactococcus lactis*, *Lactococcus plantarum*, *Lactococcus garviea*e, *Lactococcus raffinolactis* and *Lactococcus piscium*. The use of *Lactococcus* is widespread in industrial starter culture technology. Especially, *L. lactis* is widely used in fermented dairy product (Teuber, 1995).

2.3.1.4 *Leuconostoc*

The genus *Leuconostoc* is generally ovoid or cocci-shaped depending on the type of culture medium. *Leuconostoc* are separated from other cocci of the LAB by their heterofermentative metabolism. This genus produces D (-)-lactic acid, ethanol, carbon dioxide and diacetyl from glucose fermentation and have complex nutritional requirements. The range of G+C content is 37-40 %mol G+C (Dellaglio et al., 1995). *Leuconostoc*, for example *Leuconostoc mesenteroides* is the predominant genus on plants and is responsible for initiating spontaneous vegetable fermentation such as sauerkraut (Daeschel et al., 1987).

2.3.1.5 *Pediococcus*

The genus *Pediococcus*, which is the only LAB that divides in two planes to produce tetrads or pairs. This genus produces DL or L (+)-lactic acid from glucose fermentation, 0.36-1.43 micrometer in diameter and growth at 25-40°C. The range of G+C content is 34-44 %mol G+C (Simpson and Taguchi, 1995). Some species are able to produce pseudo-catalase. *Pediococcus* are important in food technology, in both a negative and positive results. Some species of this genus, for example *Pediococcus damnosus*, is a major spoilage organism in beer manufacture (Garvie, 1986b). *Pediococcus acidilactici* and *Pediococcus pentosaceus* are used as starter cultures for dry-fermented sausage and other meat products (Hammes et al., 1990).

2.3.1.6 *Enterococcus*

The genus *Enterococcus* often occurs in pairs (diplococci) or short chains. They are homofermenter, which produce L (+)-lactic acid from glucose fermentation and some species are able to produce pseudo-catalase. They can grow at 10 and 45°C and the complex nutrients are required. The range of G+C content is 37-40 %mol G+C (Devriese and Pot, 1995). *Enterococcus* are not considered important in food technology and some species, especially *Enterococcus faecium*, can be opportunistic pathogens and are generally undesirable in food (Parker, 1978).

2.3.1.7 *Vagococcus*

The genus *Vagococcus* is easily confused with *Lactococcus* but they differ from *Lactococcus* mainly in fatty acid composition and motility. Studies using 16S rRNA sequences established that *Vagococcus* was separated from the motile group N streptococci. This genus comprised 2 species such as *Vagococcus fluvialis*, which was isolated from chicken faeces and river water (Collins et al., 1989), and *Vagococcus salmoninarum*, which was isolated from diseased salmonid fish (Wallbanks et al., 1990).

2.3.1.8 *Tetragenococcus*

The genus *Tetragenococcus* contains only one species, *Tetragenococcus halophilus*, which previously regarded as *Pediococcus halophilus* (Collins et al.,1990). In addition, *T. halophilus* has a salt requirement for growth. It is able to grow in the high salt concentration at 18% NaCl. So, *T. halophilus* is an important species in the fermentation of foods containing high concentrations of salt, for example soy sauce (Garvie, 1986b).

2.3.1.9 *Aerococcus*

The genus *Aerococcus* usually divides in two planes to produce tetrads or pairs like *Pediococcus*. *Aerococcus* is microaerophilic organism, homofermenter and some species can produce pseudo-catalase. This genus comprised 2 species such as *Aerococcus urinae* and *Aerococcus* viridans, which reclassified from *Pediococcus urinae*-equi and *Pediococcus homari*, respectively (Holzapfel and Stiles, 1997)

2.3.1.10 *Oenococcus*

Oenococcus oeni, only one species of genus *Oenococcus*, which reclassified from *Leuconostoc oenos*. Dick et al. (1995) proposed the new genus *Oenococcus* because the physiological differences of *Leuconostoc oenos* from other species of *Leuconostoc*, including growth at pH 4.8 and in media containing 10% ethanol, lack of NAD-dependent glucose-6-phosphate dehydrogenase and genetic characteristics.

2.3.1.11 *Carnobacterium*

The genus *Carnobacterium* was originally classified as Lactobacilli. Later 16S rRNA sequence analysis studies of the genus *Carnobacterium* (Wallbanks et al., 1990), the results confirmed the similarity and the difference from all other LAB. The

phylogenetically of the genus is more closely related to *Enterococcus* and *Vagococcus*. They are heterofermentative and rod-shaped LAB. They have the ability to stay at low temperature as 0°C, so they are characteristically found in meat, poultry and fish (Collins et al., 1987). In addition, they can not grow at 45°C and on acetate media. The range of G+C content is 33-37.2 %mol G+C. Based on their phenotypic characteristics, the new genus *Carnobacterium* was proposed in table 2.2. Franzmann et al. (1991) purposed two additional species including *Carnobacterium funditum* and *Carnobacterium alterfunditum*.

Table 2.2 *Carnobacterium* species, their relationship to previously described bacteria and their habitat

Current nomenclature	Previous nomenclature	Habitat
C. divergens	*Lb. divergens*	Meat, poultry, surface ripened mould cheeses
C. gallinarum		Poultry
C. mobile		Poultry
C. piscicola[a]	*Lb. piscicola* *Lb. carnis* *Lb. maltaromicus*	Meat, poultry, salmonid fish
C. funditum		Antarctic lake
C. alterfunditum		Antarctic lake

[a] Proposed as *C. maltaromicus* (Collins et al., 1991).

Source: Collins et al. (1991)

2.3.1.12 *Weissella*

The genus *Weissella* is placed within the family of Leuconostocaceae. The morphology of this genus varies from spherical or lenticular cell to irregular rods. This genus comprised 7 species including *W. paramenteroides*, *W. cofusus*, *W. kandleri*, *W. minor, W. halotolerans*, *W. viridescens* and *W. hellenica*, the new species which was isolated from Sai-krok-Prew (Holzapfel and Stiles, 1997).

2.3.2 Carbohydrate fermentation of Lactic acid bacteria

All LAB can produce lactic acid from hexoses because they lack functional heme linked electron transport chains and a functional of Krebs cycle. So, they obtain energy via substrate level phosphorylation. LAB are divided into two groups based on two majors pathways of hexoses metabolization such as homofermentative LAB and heterofermentative LAB.

Homofermentative LAB refer to LAB in the group can produce lactic acid as the major or sole end-product of glucose fermentation such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some lactobacilli. Homofermenters use the Embden–Meyerhof–Parnas pathway (Figure 2.2) (Axelsson, 1998) to generate two moles of lactic acid and 2 ATP per mole of glucose consumed.

Heterofermentative LAB refer to LAB in the group can produce lactic acid , CO_2 and ethanol from glucose fermentation via phosphoketolase or 6-phosphogluconate pathway (Figure 2.3) (Axelsson, 1998) such as *Weissella* and *Leuconostoc* and some lactobacilli. From the pathway gives 1 mole each of lactic acid, ethanol, CO_2 and 1 ATP per mole of glucose.

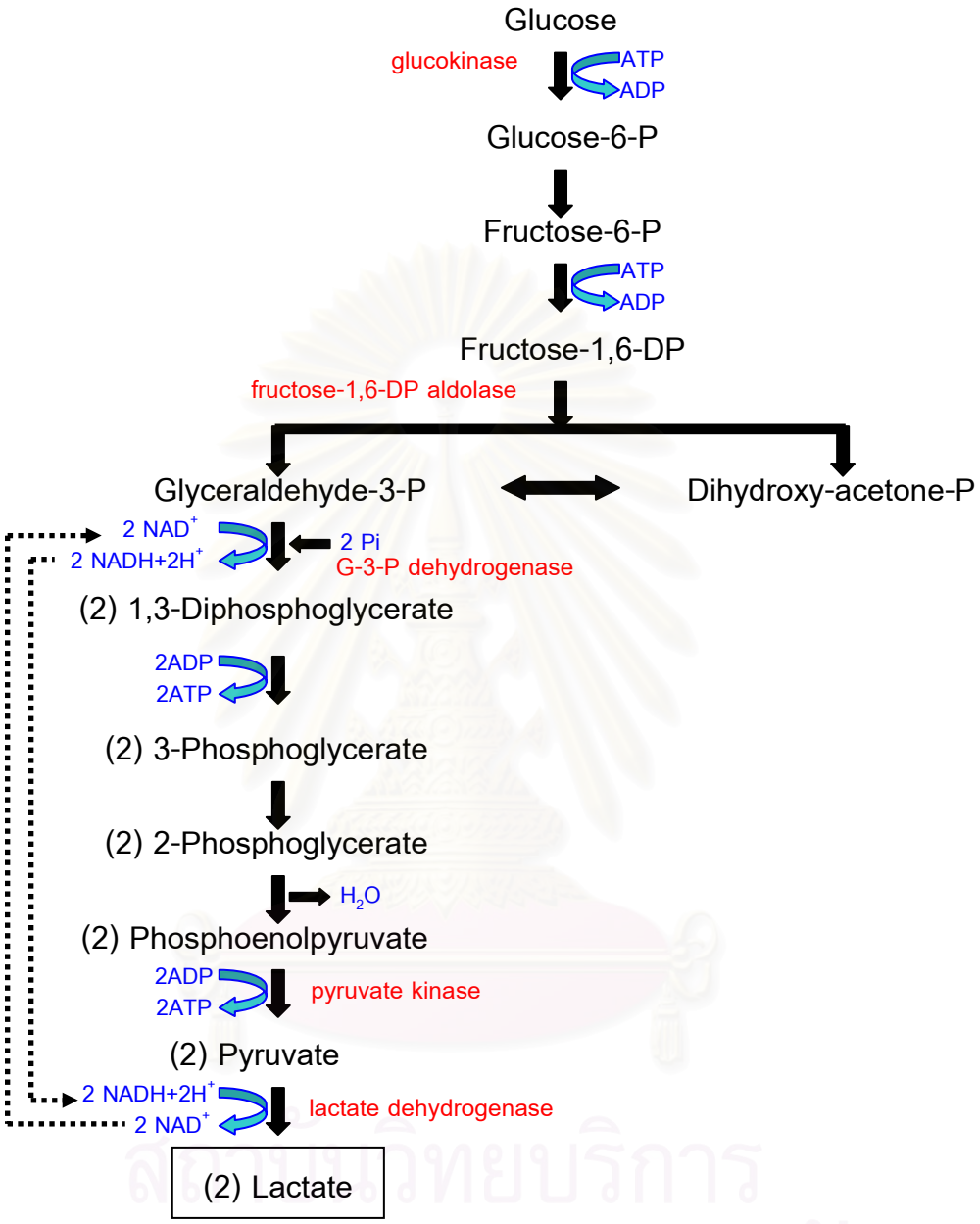


Figure 2.2 Embden–Meyerhof–Parnas pathway

Source: Axelsson (1998)

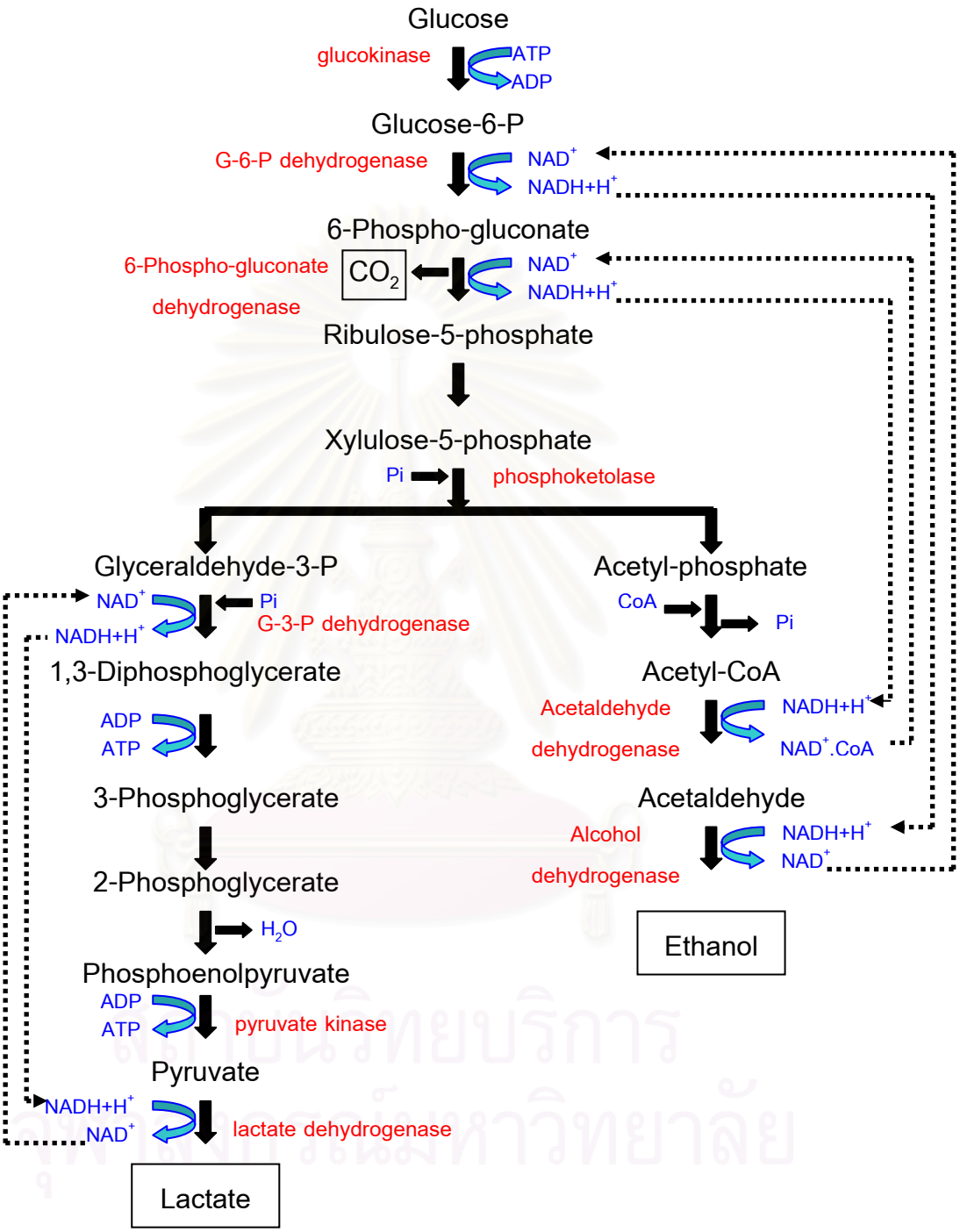


Figure 2.3 Phosphogluconate pathway

Source: Axelsson (1998)

It is well known that LAB may change their metabolism in response to differential conditions, resulting in a various end product pattern different from glucose fermentation under normal conditions. Pyruvate metabolism and the use of external electron acceptors are the other alternative ways for various product creations. The alternative fates of pyruvate are described in Figure 2.4

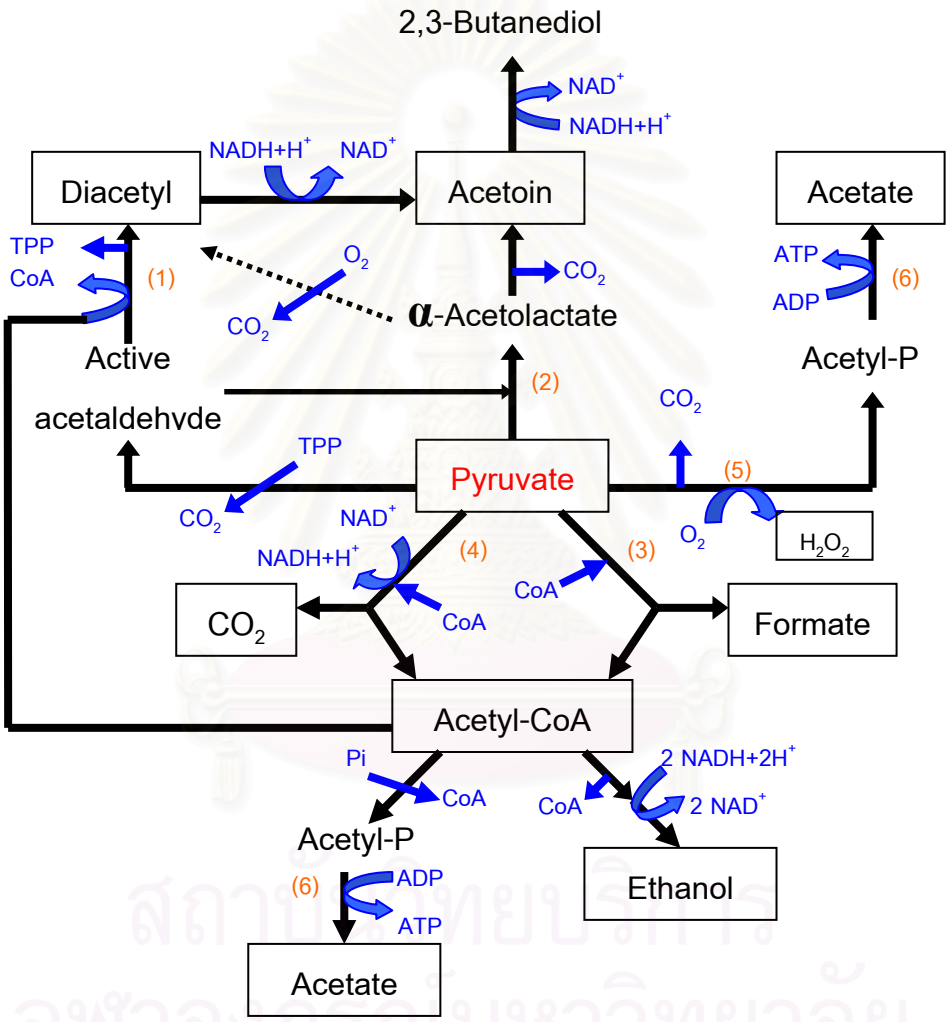


Figure 2.4 Pathways for the alternative fates of pyruvate. Dashed arrows denote a non-enzymatic reaction. Important metabolites and end products are framed. Enzymatic reactions are numbered: 1. diacetyl synthase; 2. acetolactate synthase; 3. pyruvate formate lyase; 4. pyruvate dehydrogenase; 5. pyruvate oxidase; 6. acetate kinase.

Source: Cogan and Hill (1993)

2.4 Antimicrobial substances from Lactic acid bacteria

Lactic acid bacteria (LAB) have long been used in fermentations to preserve the nutritive qualities of various foods. LAB is classified as generally recognized as safe (GRAS) and considered as 'food grade' organisms that are safe to consume (Bredholt et al., 2001). LAB is applied as starter culture or protective culture in many kinds of fermented foods like yoghurt, cheese, dry sausage, sauerkraut, and sourdough. In the meat industry, LAB is widely used as starter cultures for sausage fermentation (Liepe, 1983). They not only contribute to flavor development but also inhibit the competing natural flora, which includes spoilage bacteria and occasionally pathogen. They have the potential to be used in biopreservation due to their capacity to produce several antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocins (Jay, 1982; Klaenhammer, 1988; Piard and Desmazeaud, 1991, 1992).

2.4.1 Low-molecular-mass (LMM) compounds

2.4.1.1 Organic acids

The primary antimicrobial effect of LAB is the production of organic acids including lactic acid, acetic acid and propionic acid (Daeschel, 1989). Levels and types of organic acids produced during the fermentation process depend on various factors such as LAB species or strains, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). The direct antimicrobial effect of organic acids associates in the reduction of pH, result in the undissociated form of the organic acid molecules (Podolak et al., 1996). It has been described that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the cytoplasmic membrane and act by collapsing the electrochemical proton gradient. Moreover, the altering the cell membrane permeability which results in

disruption of substrate transport systems can be occurred. Lactic acid is the major organic acid of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH (Lindgren and Dobrogosz, 1990). Acetic acid is more inhibitory than lactic acid because yeasts, moulds and bacteria are inhibited by acetic acid. (Blom and Mortvedt, 1991). Moreover, propionic acid can inhibit fungi and bacteria and it is present in another commercial product, bioprofit where the use of a *Propionibacterium freudenreichii* strain along with *Lactobacillus rhamnosus* increases inhibitory activity against fungi and some gram positive bacteria (Måyrå-Måkinen and Suomalainen, 1995).

2.4.1.2 Hydrogen peroxide (H_2O_2)

LAB lacks true catalase to break down the hydrogen peroxide, which is produced in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The accumulation of H_2O_2 can inhibit some microorganisms. The inhibitory effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of cell proteins and membrane lipids thus increasing membrane permeability (Kong and Davison, 1980). H_2O_2 may act as a precursor for the production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl (OH^-) radicals which can damage DNA (Byczkowski and Gessner, 1988).

2.4.1.3 Carbon dioxide (CO_2)

Carbon dioxide is mainly produced by heterofermentative LAB. CO_2 can directly create an anaerobic environment, which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund, 1984). Moreover, the effect of CO_2 can cause the growth inhibition of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria (Farber, 1991).

2.4.1.4 Diacetyl

Diacetyl, an aroma compound, is produced by many LAB including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Lactobacillus* by citrate metabolism (Cogan and Hill, 1993). Gram-negative bacteria, yeasts and moulds are more sensitive to diacetyl than gram-positive bacteria. The inhibitory effect of diacetyl is associated with the interference of arginine utilization (Jay, 1986)

2.4.1.5 Reuterin

Reuterin is an antimicrobial substance, which is a low-molecular mass and non-proteinaceous component. Lindgren and Dobrogosz (1990) reported that reuterin is produced during stationary phase by the anaerobic growth of *Lactobacillus reuteri* on a mixture of glucose and glycerol or glyceraldehyde. Antimicrobial spectrum of reuterin is affected for bacteria, viruses, fungi and protozoa (Axelsson et al., 1989; Chung et al., 1989). The mode of action of reuterin is associated with the inhibition of ribonucleotide reductase, which is the essential enzyme for DNA-synthesis (Dobrogosz et al., 1989).

2.4.2 High-molecular-mass (HMM) compounds

Bacteriocins have more attractive in food industry due to their applicational potentiality in food preservation. Bacteriocins are ribosomally-synthesized antimicrobial polypeptides or proteins, extracellularly released, which have a bactericidal or bacteriostatic effect against a narrow spectrum of closely related species (Jack et al., 1995). The producer cell exhibits a specific immunity to the action of its own bacteriocin. The target of bacteriocins is the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipids bilayer (Montville et al.,1995). The mode of action of bacteriocins is shown in figure 2.5. Because of the protective barrier provided by the lipopolysaccharides (LPS) of the outer membrane of Gram-

negative bacteria, they are generally only active against Gram-positive bacteria (Abee et al., 1995).

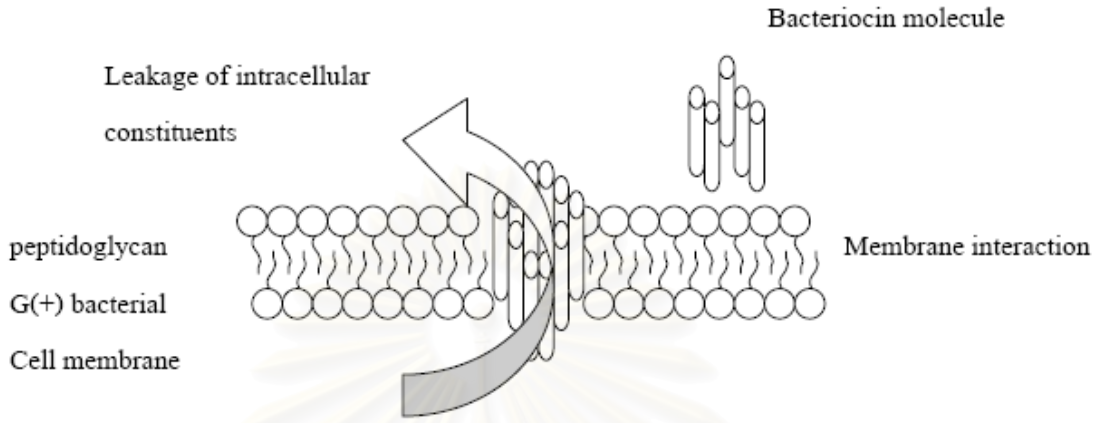


Figure 2.5 The mode of action of bacteriocins that cause the pore formation on the cytoplasmic membrane.

Source: Ruhr et al. (1985)

Bacteriocins from LAB may be divided into three classes (Nes et al., 1996) based on molecular weight, biochemical properties, range of sensitive hosts and mode of actions.

Class I : Bacteriocins of this class contain post-translationally modified amino acids and are also termed lantibiotics. These are small and heat-stable peptides that have been differentiated from other bacteriocins by containing dehydroamino acids and thioether amino acids like lanthionine and/or ß-methyl- lanthionine residues. The most studies of this class is nisin, which is produced by *Lactococcus lactis* subsp. *lactis* and is GRAS status for using directly in human food. Nisin contains 34 amino acids, 5 thioether bonds, 3,500 daltons of molecular weight (Gross and Morell, 1967) and has a broad inhibitory spectrum against gram-positive bacteria and can prevent outgrowth of *Bacillus* and *Clostridium* spores (Daeschel, 1989).

Class II : Bacteriocins in this classes are heat-stable peptides, non-lantibiotics and low molecular mass (<10 kDa), which can divide into three subclasses (table 2.3) on the basis of either their distinctive N-terminal sequence, their formation of bicomponent pores, or the presence of a functional sulfhydryl group. Class IIA is the most common and seems to involve disruption of mannose transport into target cells. This group comprises single peptide bacteriocins including pediocin-like bacteriocins with anti-listerial activity, which are produced by *Pediococcus* spp. Pediocin A, Pediocin PA-1 and Pediocin Ach are produced by *Pediococcus pentosaceus*, *Pediococcus acidilactici* PAC 1.0 and *Pediococcus acidilactici* H, respectively (Gonzales and Kunka, 1987; Bhunia et al., 1988). Class IIb comprises double-peptide bacteriocins, which form pores in the membranes of target cells and disrupt the proton gradient of target cells. Other bacteriocins can be grouped together as Class IIc, the sec-dependent secreted bacteriocins, which have a wide range of effects on membrane permeability, cell wall formation and pheromone actions of target cells (Von Heijne, 1986, 1988).

Table 2.3 Classes of bacteriocins produced by lactic acid bacteria

Class	Subclass	Description
I		Lantibiotics-small, heat stable, containing unusual amino acids
II		Small (30-100 amino acids), heat stable, non-lantibiotic
	IIa	Pediocin-like bacteriocins, with anti-listerial effects
	IIb	Two peptide bacteriocins
	IIc	Sec-dependent secretion of bacteriocins
III		Large (> 30 kDa) heat-labile proteins

Source : Reproduced from Daly et al. (1998)

Class III : Bacteriocins in this classes are heat-labile peptides and large molecular mass (> 30 KDa), which include many bacteriolytic extracellular enzymes

(hemolysins and muramidases) that may imitate the physiological activities of bacteriocins.

2.5 The isolation source of bacteriocin-producing lactic acid bacteria

Osmanağaoğlu et al. (2001) isolated *Pediococcus pentosaceus* Pep1 from vacuum-packed sausages. It exhibited a potentially novel antimicrobial agent active against food spoilage following *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, *Enterococcus*, *Bacillus* and *Listeria*. It could not inhibit Gram-negative bacteria such as *Yersinia enterocolitica* and *Escherichia coli*. The antimicrobial agent was sensitive to proteolytic enzymes, resistant to heat and organic solvents and active over wide range of pH values between 3 and 8. It was identified as a bacteriocin in termed pediocin P.

De Martinis et al. (2003) screened for bacteriocin-producing LAB comprised twenty samples of vacuum-packaged meat products from Brazil by using an agar overlay method. Three bacteriocinogenic LAB were isolated and identified as *Enterococcus* sp. 18 (from bacon), *Leuconostoc* sp. 20 (from ham) and *Lactobacillus sakei* 29 (from hot home made “lingüiça”). *Leuconostoc* sp. 20 and *Lactobacillus sakei* 29 exhibited antilisterial activity that inhibited all the strains of *Listeria monocytogenes* tested. However, *Brochothrix thermosphacta*, *Enterobacter* sp., *Salmonella* sp. and *Escherichia coli* were not inhibited by the strains.

Noonpakdee et al. (2003) isolated a total of 14,020 LAB from nham, a Thai traditional fermented pork sausage, and screened for bacteriocin production. Only one *Lactococcus lactis* strain WNC 20 was able to produce a bacteriocin that not only inhibited closely related LAB, but also some food-borne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus*. The study of α-chymotrypsin and proteinase K treatment, Biochemical tests and the spectrum of antimicrobial substance were nearly identical to nisin. Sequencing of gene

encoding this bacteriocin was studied. The result showed identical sequences to nisin Z. Therefore, the ability of the bacteriocin produced by *L. lactis* WNC 20 may be useful and apply for improving the food safety of the fermented products.

Srionnual et al. (2007), isolated *Weissella cibaria* 110 which produced bacteriocin active against some Gram-positive bacteria from plaa-som, a Thai fermented fish product. The exposure of high temperature and catalase enzyme was not affected the bacteriocin activity, but the inactivation of bacteriocin occurred after the treatment of proteolytic enzymes. The molecular weight of the bacteriocin was about 2.5 kDa according to SDS-PAGE analysis. Mass spectrometry analysis showed the mass of the peptide approximately 3,487.8 Da. N-terminal amino acid sequence analysis was identified 27 amino acids that no similarity to other known bacteriocins. This bacteriocin was defined as a new bacteriocin in termed weissellicin 110.

Ponce et al. (2008) isolated a total of 45 LAB from organic leafy vegetables, and investigated by its antimicrobial activity. Selected isolates were identified as *Enterococcus faecium*, *Lactococcus lactis*, *Enterococcus hirae* and *Enterococcus canis.* Bacteriocin-like substances were active against Gram-positive and Gram-negative foodborne pathogens including *Listeria monocytogenes* and *Escherichia coli*, respectively. In all four strains, the bacteriocin activity was stable after extended refrigerated storage and freezing-thawing cycles. In contrast, the inactivation of bacteriocin occurred after the treatment of proteolytic enzymes. This experiment suggested that four LAB strains may be applied as biopreservatives in minimally process vegetables.

Shin et al. (2008) isolated a total of 1,000 LAB from various Kimchi samples, Korea traditional fermented vegetables, and screened for the bacteriocin production. Pediocin K23-2, a bacteriocin produced by the *Pediococcus pentosaceus* K23-2 strain, showed strong inhibitory activity against Gram-positive bacteria, especially *Listeria monocytogenes*. The bacteriocin is heat stable. However, it inactivated after treatment

with proteolytic enzymes. The molecular weight of the bacteriocin was about 5 kDa according to a tricine SDS-PAGE analysis. From this experiment, *P. pentosaceus* K23-2 isolated from Kimchi produces a bacteriocin, which shares similar characteristics to the Class IIa bacteriocins.

2.6 Application of lactic acid bacteria in meat products

2.6.1 Market problems of nham

The marketing problems of traditional nham include its short shelf life and high price and the intensive labor required for its production. It has high energy costs if kept under refrigeration in the marketplace. In addition, the manufacturers have a heavy exposure to risk of losing a large stock through a process failure. Pork meat is quite expensive, and the raw material cost is increasing more quickly than the selling price. Moreover, large-scale production of nham has the problem of its short storage life because nham has high moisture content. A longer shelf life is required so that the nham can be distributed to the marketplace. Therefore, the nham market needs the product to have consistent quality, safety, and longer shelf life. The nham should stay fresh and not turn rancid or develop an off flavor or change in color when it is in the marketplace.

Generally, the fermentation of nham is spontaneous by indigenous microorganisms from raw materials. The suitable acid production is important to contribute both of quality and safety of products, but the product quality varies from batch to batch. The shelf life of nham is quite short - approximately a week at ambient temperatures. The chilled-storage can extend the shelf life, but normally the product is stored at ambient temperatures. Furthermore, the sanitation of the process is also poor because of a lack of knowledge and technology. The native lactic acid bacteria, which are the dominant microorganisms (Tanasupawat and Daengsubha, 1983), may be insufficient to produce the proper organic acids, so the pH value of products can not drop to 4.4-4.8, which is usually pH of nham (Phithakpol et al, 1995). This condition may

allow pathogenic bacteria to grow before lactic acid bacteria occur, resulting in the possibility of food poisoning. Recently, consumers prefer more stable and safer products with a longer shelf life and without chemical preservatives. In addition, most nham is consumed without further cooking and proper processes for the production of nham should be developed for ensuring that acid production is adequate to lower the pH of the end products. To achieve for this purpose, the application of starter culture technology in the production of nham is a way of natural food preservation.

2.6.2 Biopreservation

In 1996, Jay reviewed the concept of microbial interference and described as the antagonism displayed by one microorganism towards another that a concept is associated with lactic acid bacteria (LAB). This concept is like the meaning of the protective cultures and the bioprotection concepts of Holzapfel et al (1995) and Stiles (1996). According to the latter author, biopreservation refers to extend storage life and enhanced safety of foods using their natural or controlled microflora and (or) their antibacterial products.

Biopreservation can be applied in food and meat systems by four basic methods (Stiles, 1996; Gorris, 1997).

- Adding a pure culture of the viable bacteriocin-producing LAB, which is an indirect way to take bacteriocins in food products. The ability of the culture to grow and produce bacteriocins in the food under the environmental and technological conditions (temperature, pH, additives, etc.) are very important for its success. As meat cannot be pasteurized prior to the addition of a LAB culture, the LAB cultures for biopreservation or fermentation of meat must be able to compete with the natural microflora.

- Adding a crude bacteriocin-preparation, the fermentation liquor or concentrates obtained from the growth of bacteriocin-producing LAB in a complex substrate. This mode avoids the use of a purified compound.

- Adding purified or semi-purified antagonistic substances. By using this method, the dosage of bacteriocin is more accurate and thus more predictable. However, application is limited according to national regulations concerning food additives.

- Adding mesophilic LAB as a 'fail-safe' protection against abuse of temperature. In this case the bioprotective strain will be kept at initial concentration in chilling conditions. Under temperature-abuse conditions, the strain will grow competitively in front of pathogenic bacteria avoiding health hazards.

2.6.3 The type of starter cultures or protective cultures

Protective cultures can be divided in two types:

2.6.3.1 Non-bacteriocinogenic cultures

The antagonistic character of non-bacteriocinogenic protective cultures are based on the competition for nutrients and product acidification through lactic acid production resulting in the growth retardation of spoilage and (or) pathogenic bacteria (Juven et al., 1998).

Daly et al. (1970) demonstrated the inhibition of the food-borne pathogens *Pseudomonas fluorescens*, *Staphylococcus aureus* and *Clostridium perfringens* by inoculation with the starter *Streptococcus diacetylactis*. This study showed that *S. aureus* populations were reduced more than 99% in food model such as ham sandwich

spread, chicken gravy and ground beef, and that the inhibition was most likely as a result of acid production by *S. diacetylactis*.

Vermeiren, Devlieghere and Debevere (2006) studied the interaction of non-bacteriocinogenic , *Lactobacillus sakei* subsp. carnosus (10A) and lactocin S producing *Lactobacillus sakei* 148 (LS5) towards a cocktail of three *Listeria monocytogenes* strains on a model cooked ham (MCH). The influence of inoculation level (10^5 and 10^6 CFU/g), storage temperature (4 and 7 °C) and packaging type (vacuum-packaging and modified atmosphere packaging) were examined. The results showed that an application of 10^6 $CFUg^{-1}$ of strain 10A at 7°C and under vacuum-packaging limited the increase of *Listeria monocytogenes* to lower than 1 $log_{10}CFUg^{-1}$ during 27 days, while an application level of 10^5 $CFUg^{-1}$ failed to prevent growth to unacceptable levels. In the other hand, strain LS5 was unable to inhibit the growth of *L. monocytogenes*. The temperature 4 or 7°C and the packaging type did not influence the growth of strain 10A on the MCH, as its dominance did not change. Sensory assessments and pH measurements confirmed that 10A, even when present at a high level for prolonged storage times, did not acidify the MCH to a point of sensory rejection. Therefore, the antagonistic activity towards *L. monocytogenes* cannot be the result of lactic acid production and the associated pH decrease. The mechanism of the inhibition is probably occurred on the production of more complex antimicrobials and competition or depletion of specific nutrients might explain the protective effect of this culture.

Riebroya et al. (2008) investigated the effect of inoculation of different lactic acid bacteria (LAB) on the fermentation and quality of Som-fug, a Thai traditional fermented fish from bigeye snapper. Som-fug inoculated with *Pediococcus acidilactici* at 10^4 $CFUg^{-1}$ (PA104) had a greater acceptability than those inoculated with *Lactobacillus plantarum* and *Pediococcus pentosaceus* at either 10^4 or 10^6 $CFUg^{-1}$ and the control (without inoculum). PA104 exhibited a higher rate of fermentation than the control as indicated by the greater rate of pH drop and lactic acid production. Based on pH desired at 4.5, the fermentation was completed within 48 and 36 h for the control and

PA104, respectively. Som-fug inoculated with PA104 exhibited higher hardness and adhesiveness than the control. From the result, the inoculation of *P. acidilactici* was able to decrease the fermentation time and improve the quality of Som-fug. Therefore, *P. acidilactici* can be used as a potential starter for Som-fug fermentation.

Bredholt et al. (1999) studied the use of indigenous LAB as protective cultures for the growth inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in cooked meat products. The five LAB strains were isolated from commercial products and identified as *Lactobacillus sakei*. From the results, *L. sakei* inhibited the growth of both *L. monocytogenes* and *E. coli* O157:H7. The inhibitory effect on *Yersinia enterocolitica* O:3 was not observed. In addition, all samples had acceptable sensory properties. These indigenous LAB may be used as protective cultures to inhibit growth of *L. monocytogenes* and *E. coli* O157:H7 in cooked meat products.

2.6.3.2 Bacteriocinogenic cultures

The antagonistic character of bacteriocinogenic protective cultures is based on the production of one or more bacteriocins or bacteriocin-like compounds. There has been a dramatic increase in the number of novel bacteriocins discovered in the last decade, but the potential application is limited by some properties such as spectrum of inhibition, heat stability and solubility. In general, the selection of bacteriocin-producing strains for food application should be considered following by Holzapfel et al. (1995)

- The producing strain should have a GRAS status
- Have a broad spectrum of inhibition which includes pathogens such as *Listeria monocytogenes* and *Clostridium botulinum*, or specificity against a particular pathogen
- Have a high specific activity
- Heat stable and no associated health risks

- Lead to beneficial effects in product such as improved safety, quality and flavour

Bennik et al. (1999) studied the potential to control the growth of *Listeria monocytogenes* on refrigerated and modified atmosphere (MA) stored mungbean sprouts of Two bacteriocinogenic strains of *Pediococcus parvulus* and one bacteriocinogenic *Enterococcus mundtii* strain. Only *Ent. mundtii* was capable of bacteriocin production at 4–8 °C. *Ent. mundtii* was subsequently evaluated for its ability to control the growth of *L. monocytogenes* on vegetable agar and fresh mungbean sprouts under 1·5% O_2 : 20% CO_2 : 78·5% N_2 at 8 °C. The growth of *L. monocytogenes* was inhibited by bacteriocinogenic *Ent. mundtii* on sterile vegetable-medium but not on fresh produce. However, mundticin, the bacteriocin produced by *Ent. mundtii*, was found to have potential as a biopreservative agent for modified atmosphere stored mungbean sprouts when used in a washing step or a coating procedure.

Benkerroum et al. (2005) studied the effectiveness of *in situ* bacteriocin production by lactic acid bacteria (LAB) to control *Listeria monocytogenes* in dry-fermented sausages. The lyophilized of two bacteriocin-producing strains: *Lactococcus lactis* subsp. *lactis* LMG21206 and *Lactobacillus curvatus* LBPE (Bac+ starter) were prepared and mixed with a commercial starter culture (Bel'meat^{TM} SL-25) not inhibitory to *L. monocytogenes* (Bac$^-$ starter). The experimental fermented sausages were contaminated with a mixture of four different strains of *L. monocytogenes* at level 10^2–10^3 $CFUg^{-1}$. The results showed that the cell counts of *L. monocytogenes* decreased to below the detectable limit (lower than10 $CFUg^{-1}$) after 4 hours of fermentation and no survivors could be recovered by enrichment beyond day 8 of drying, when the Bac+ starter contained *Lb. curvatus* LBPE. In the other hand, the cell counts of *L. monocytogenes* decreased to below the detectable limit was achieved after 15 days of drying, when the Bac+ starter culture containing *Lc. lactis* LMG21206 was used.

Budde et al. (2003) studied the potential of leucocin A and leucocin C - producing *Leuconostoc carnosum* 4010, when used as a protective culture for the growth inhibition of *Listeria monocytogenes* in vacuum-packed meats. The results showed that the addition of 10^7 $CFUg^{-1}$ *Leuc. carnosum* 4010 to a vacuum-packaged meat sausage immediately reduced the number of viable *L. monocytogenes* cells to a level below the detection limit and no increase of *L. monocytogenes* was observed during storage at 5 °C for 21 days. Therefore, *Leuc. carnosum* 4010 is suitable as a new protective culture for cold-stored, cooked, sliced, and vacuum-packed meat products.

2.7 Foodborne and Pathogenic bacteria

Pork meat is a mainly composition of nham production that provide an excellent nutrient and environment for the growth of pathogenic and foodborne-organisms. Therefore, food-poisoning diseases by consuming a contaminated of fermented meat products has been concerned. The food-poisoning are caused by viruses, bacteria, parasites and toxins-produced from some microorganisms. However, the most serious type of food poisoning is bacterial food poisoning, which includes foodborne intoxications and infections. The most common bacteria that respond to food intoxications include *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens* and *Clostridium botulinum*. In addition, *Salmonella*, *Listeria monocytogenes*, *Shigella Campylobacter jejuni* and *Vibrio parahaemolyticus* are the common bacteria that respond to food infections.

2.7.1 *Bacillus cereus*

Bacillus cereus is a Gram-positive, rod-shaped, beta-hemolysin producer and aerobic or facultative aerobic spore-forming bacteria that widely distributed in the environment, mainly in soil (Becker et al., 1994). It orders in the Bacillaceae family. Furthermore, it is easily contaminated many types of foods, especially those of vegetable origin, however, it is also frequently isolated from meat, eggs and dairy

products (Kramer and Gilbert, 1989). *B. cereus* causes two types of food-borne illnesses. It produces one emetic toxin (ETE) and at least three different enterotoxins (HBL, Nhe and EntK), which are responsible for separate emetic and diarrheal syndromes, respectively.

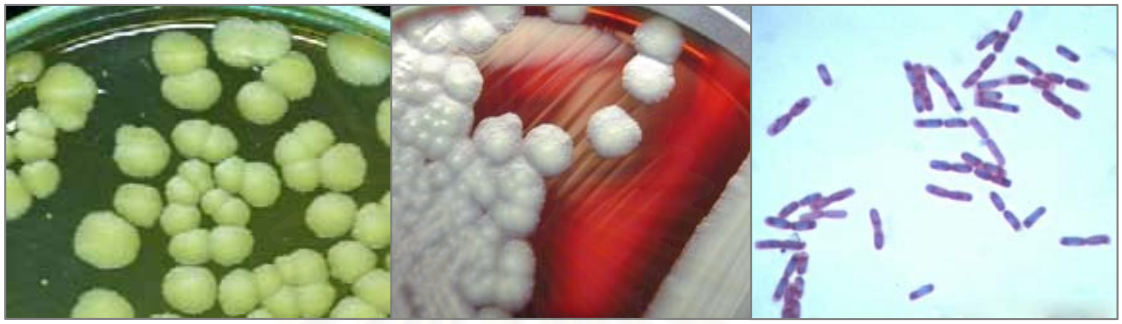


Figure 2.6 Colonial appearance of *Bacillus cereus* on Tryptic Soy agar (left), on sheep-blood agar (middle) and Gram staining (right)

Available from:

http://atlas.medmicro.info/obrazek.php?id=1 [2009, February 3]

http://wpcontent.answers.com/wikipedia/commons/thumb/0/02/Bacillus_cereus_01.png/240px-Bacillus_cereus_01.png [2009, February 3]

- **Emetic syndrome** is caused by a toxin called cereulide that is found only in emetic strains. This syndrome is associated with the consumption of rice, pasta, pastry, and noodles in which *B. cereus* has grown and produced the toxin that leads to nausea and vomiting. It has an incubation period of 1 to 6 hours after consumption. This type is called the "short-incubation" or emetic form, that its symptoms and incubation period resembles *Staphylococcus aureus* food poisoning (Lund and Granum, 1997; Turnbull, 1979).

- **Diarrheal syndrome** is caused by the enterotoxins produced from *B. cereus* during vegetative growth in the small intestine, and a wide variety of foods, including meat and vegetable dishes, soups, and dairy products (Granum, 1994). Two of the three enterotoxins are involved in food poisoning, which is associated with diarrhea and

gastrointestinal pain (Gilbert, 1979). One of these enterotoxins is HBL, which is also a hemolysin. The second enterotoxin is Nhe, which is not a hemolysin. The third enterotoxin is EntK, which is a single component protein that has not been shown to be involved in food poisoning. All three enterotoxins are cytotoxic and cell membrane active toxins that will make holes or channels in membranes. It has an incubation period of 8 to16 hours. This type is called the "long-incubation" or diarrheal form, that it resembles food poisoning caused by *Clostridium perfringens* (Gilligan, 1983)

2.7.2 *Listeria monocytoge*nes

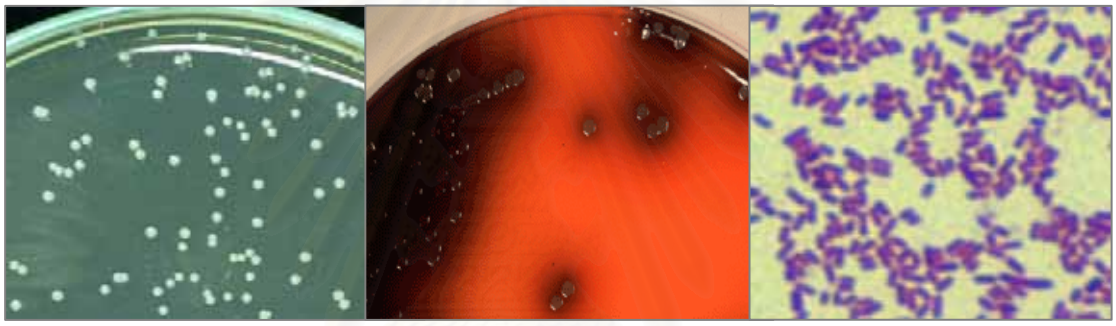


Figure 2.7 Colonial appearance of *Listeria monocytogenes* on Tryptic Soy agar (left), on PALCAM medium base agar (middle) and Gram staining (right)

Available from:

http://www.biotec.com/images/PalcamAgar.gif [2009, February 3]

http://hillagric.ernet.in/education/covas/vph/images/Lis.JPG [2009, February 3]

Listeria monocytogenes is a Gram-positive, non-spore forming, motile, facultatively anaerobic and rod shaped bacteria. It is catalase positive, oxidase negative, and expresses a beta-hemolysin. It orders in the listeriaceae family. It is ubiquitous in the environment that can be isolated from wild and domestic animals, birds, insects, soil, wastewater, and vegetation. *L. monocytogenes* is an opportunistic foodborne pathogen that caused the serious disease listeriosis. The disease infects primarily in pregnant women, elderly, and newborns (Schlech, 2000; Tauxe, 2002), that

can cause septicemia, meningitis (or meningoencephalitis), encephalitis, corneal ulcer, pneumonia and spontaneous abortion or stillbirth (Gray and Killinger, 1966).

Food-borne outbreaks of human listeriosis have occurred after the consumption of contaminated food including meat, poultry-meat, fish products (frankfurters, pâté, smoked salmon, fermented raw meat sausages), dairy products (soft cheeses, unpasteurised milk) and prepared salads (coleslaw, bean sprouts) (Ryser, 1999). Because of the ability of survival and adaptation for environmental stress conditions such as low pH, low water activity, heat, refrigeration temperatures, sodium chloride and sodium nitrate that contributes significantly to its hazard status (Vignolo et al., 1996), so its contamination in food products is a serious safety problem in the food industry.

2.7.3 *Staphylococcus aureus*

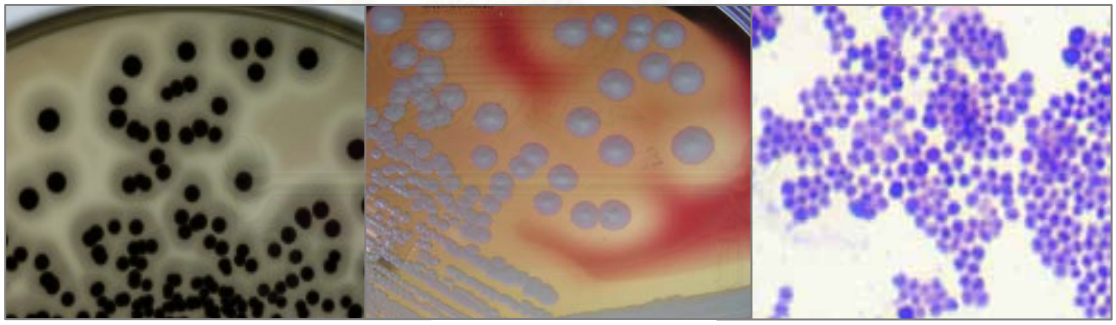


Figure 2.8 Colonial appearance of *Staphylococcus aureus* on Baird Parker agar (left), on sheep-blood agar (middle) and Gram staining (right)

Available from:

http://www.labm.com/images/news_press_releases/t54_1.jpg [2009, March 2]

http://www.arabslab.com/vb/showthread.php?t=8567&page=4 [2009, March 4]

http://www.gslabs.com/images/saureus2.jpg [2009, March 4]

Staphylococcus aureus is a Gram-positive, cocci shaped, non-motile, positive catalase, positive coagulase and facultative anaerobic bacteria. It orders in the

staphylococcaceae family. *S. aureus* is a flora organism that present on the skin and mucosae of humans and animals and is ubiquitous in the environment (Jay, 1997). It is commonly present in some processing plants, such as poultry processing lines (Notermans et al., 1982, 1983; Mead et al., 1989). *S. aureus* has been isolated from several foods such as meat, chicken, dairy products, fermented food, vegetables and fish products (Wieneke et al., 1993; Tamarapu et al., 2001).

It is able to produce several enterotoxins (SEs) such as SEA, SEB, SEC, SED, SEE, SEG and SEH (Van den Bussche et al., 1993) that cause intoxication symptoms in humans. SEA is the most enterotoxin that recovered from food-poisoning outbreaks and followed by SED and SEB (Casman, 1965). SEs are resistant to inactivation by gastrointestinal proteases such as pepsin and heat. Heat stability is one of the most important properties of SEs in terms of food safety (Denny et al., 1971; Hernandez et al., 1993).

2.7.4 *Salmonella*

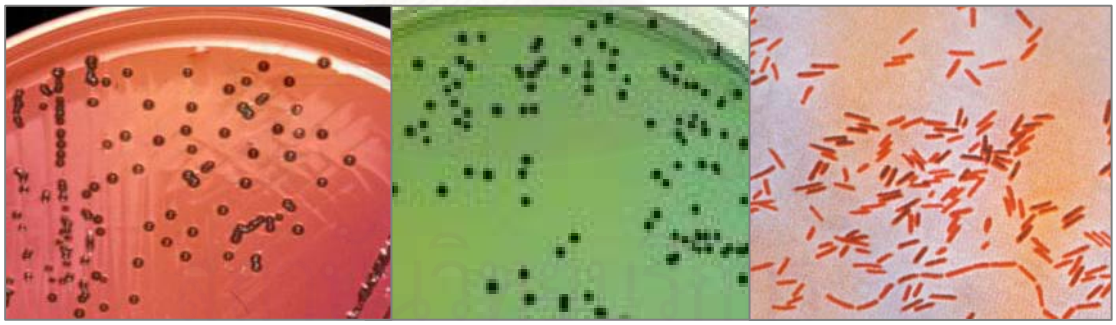


Figure 2.9 Colonial appearance of *Salmonella* on Xylose Lysine agar (left), on Hecktoen enteric agar (middle) and Gram staining (right)

Available from:

http://bioinfo.bact.wisc.edu/themicrobialworld/Salmonella.XLD.jpg[2009, March 5]

http://www.podloza.pl/resources/files/870_1.jpg[2009, March 5]

http://bioinfo.bact.wisc.edu/themicrobialworld/S.typhi.Gram.jpg [2009, March 5]

Salmonella is a Gram negative bacteria that has rod- shaped. It orders in the enterobacteriaceae family. It is usually able to motile by peritrichous flagella and to grow in aerobic or facultatively anaerobic condition (Minor, 1984). Moreover, hydrogen sulfide is produced by *Salmonella* when it grows in suitable medium. *Salmonella* is a pathogenic bacteria for humans that causes typhoid fever, paratyphoid fever and the foodborne illness salmonellosis (Ryan and Ray, 2004). *Salmonella* is widespread in the environment and appear in a wide variety of foods and food ingredients that is a great problem to the food industry. The incidence of *Salmonella* in poultry has been well determined in many countries. The serovars isolated vary geographically, but frequently include *Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Virchow and *Salmonella* Typhimurium (Foster, 1969).

Salmonellosis is the most prevalent foodborne disease in many countries worldwide that caused by eating contaminated food with *Salmonella*. The symptoms of salmonellosis usually appear about 12 to 36 hours after eating contaminated food. Abdominal pain, diarrhea, nausea, vomiting, chills, and fever are common symptoms that found in people who infected with *Salmonella*. In addition, dehydration, headache and prostration also may occur. The elderly, infants, and those with impaired immune systems are more likely to have a severe illness. The severity and duration of symptoms depend on the type of *Salmonella* present, the amount of food eaten, and the susceptibility of the person involved (Gravani, 1984).

2.7.5 *Escherichia coli*

Escherichia coli is a Gram negative bacteria that has rod-shaped and non-sporulating. It orders in the enterobacteriaceae family. It is usually able to motile by peritrichous flagella and to grow in aerobic or facultatively anaerobic condition (Darnton, 2007). It normally presents as normal flora in gastrointestinal tract of humans and is able to find in food or water. Moreover, *E. coli* is used as indicator organisms to test the effectiveness of effluent disinfection in a wastewater treatment plant.

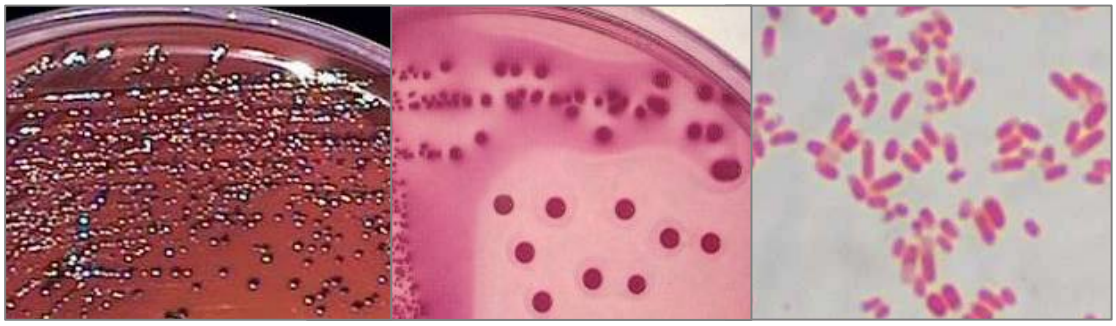


Figure 2.10 Colonial appearance of *Escherichia coli* on Eosin Methylene Blue agar (left), on MacConkey agar (middle) and Gram staining(right).

Available from:

http://www.nmconline.org/images/ecoli.jpg[2009, March 5]

http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Gram_stain_images/E_coli_2000_P7201172.jpg[2009, March 5]

http://www.ams.cmu.ac.th/mt/clinmcrb/CMBwebsite/Price%20list_media.htm. [2009, March 5]

Table 2.4 The important serotypes of diarrheagenic *E.coli*

Pathogenic groups	Common serogroups
Enteropathogenic *E.coli* (EPEC)	O26, O55, O86, O111, O114, O125, O126, O127, O128, O142
Enterotoxigenic *E.coli* (ETEC)	O6, O8, O15, O25, O27, O63, O119, O125, O126, O127, O128
Enteroinvasive *E.coli* (EIEC)	O78, O115, O148, O153, O159, O167
Enterohaemorrhagic*E.coli* (EHEC)	O26, O111, O113, O124, O136, O143, O144, O152, O157, O164
Enteroaggregative *E. coli* (EAEC)	> 50 O serogroups

Source: Chart (2002)

Although *E. coli* play the role of microorganisms flora, but some strains of *E. coli* has a virulent factor that can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome (HUS), peritonitis, mastitis, septicemia and Gram-negative pneumonia. Some foods that can cause *E. coli* food poisoning include undercooked ground beef,

vegetables and non-pasteurized fruit juice. Recently, *E. coli* is classified on the basis of serological characteristics and virulence properties following Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAEC) (Tortorello, 2000).

Enterotoxigenic *E. coli* (ETEC) is a common cause of diarrheal disease without fever in infants and travelers in underdeveloped countries or regions of poor sanitation. In a survey of the prevalence in Thailand, ETEC was wide spread in suckling pigs, water sources on pig farms and pig handlers. ETEC can produce two proteinaceous enterotoxins including the high molecular weight LT (heat-labile) toxin and the low molecular weight ST (heat-stable) toxin. The LT enterotoxin is very similar to cholera toxin of *Vibrio cholerae* in both structure and mode of action. In contrast, the action of ST enterotoxin has yet to be determined (Evan et al., 1997).

From table 2.4, *E. coli* 0157:H7 is one of the enterohemorrhagic *E. coli* (EHEC) serotypes, that is a human pathogen associated with meat and meat products, dairy products, vegetables, and water (Browning et al., 1990; Galane and Le Roux, 2001; Obi et al., 2004; Magwira et al., 2005). The diseases from this strain including uncomplicated diarrhea to hemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic Purpura (TTP) (Kaper, 1998), that caused by the consumption of *E. coli* O157:H7 contaminated food. *E. coli* O157:H7 is able to produce verocytotoxins, that known as shiga-like toxins or shiga toxins (Tsch.ape and Fruth, 2001). Verocytotoxins are known as stx1 and stx2 and divided into two main groups (VT1 and VT2) based on their antigens. Their ability of toxins are able to inhibit protein synthesis in eukaryotic cells and to induce a characteristic cytopathic effect on monolayers of VERO cells (Nataro and Kaper, 1998).

Enteropathogenic *E. coli* (EPEC) is the most important pathogens that associated with the infection in children less than 2 years of age. It can cause infantile diarrhea in developing countries (Afset et al., 2003). There are three stages in the

interactions between EPEC and host cells following (Chen and Frankel, 2005; Garmendia et al., 2005; Nataro and Kaper, 1998; Vallance and Finlay, 2000).

- adherence to epithelial cells of the host cell
- production and translocation of bacteria proteins through a needle complex via a type III secretory system.
- intimate bacterial attachment with pedestal formation

The major virulence proteins of EPEC is adhesin known as intimin, which is necessary for the bacteria attachment to the epithelial host cells (Chen and Frankel, 2005; Vallance and Finlay, 2000).

Enteroaggregative *E. coli* (EAEC) is associated with adherence factors and toxins production. It is recognized by the characteristic aggregative adherence (AA) or "stacked-brick" adherence (Nataro, et al., 1987). In addition, it can produce a hemolysin and ST enterotoxin similar to that of ETEC. The infection with EAEC strains is associated with watery, mucoid and secretory diarrhea, low-grade or absence of fever and some times vomiting (Bhan, et al., 1989b; Paul, et al., 1994).

Enteroinvasive *E. coli* (EIEC) is an important strain that mainly caused of watery diarrhea, dysentery in young children in less-developed countries. Moreover, it caused of foodborne outbreaks in adults in industrialized countries. The syndromes of EIEC closely related to the infection of *Shigella* spp. (Snyder et al., 1984; Taylor et al., 1986; Levine, 1987) that can results in high fever, malaise, abdominal cramps and overt dysentery. It mainly transmitted from contaminated food and water. The cytotoxins and enterotoxins are involved in pathogenesis that caused by EIEC. The ability of EIEC is capable to invade and multiply within epithelial cells of distal small intestine and the colon (DuPont et al., 1971).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Chemicals

Media used in this research are listed as follows:

1. Bacto Agar, purchased from Difco Laboratories, U.S.A.
2. Bacto Tryptone, purchased from Difco Laboratories, U.S.A.
3. Bacto Peptone, purchased from Difco Laboratories, U.S.A.
4. Baird-Parker Agar Base, purchased from Difco Laboratories, U.S.A.
5. Beef extract, purchased from LAB-SCAN Analytical Sciences, Thailand.
6. Bismuth Sulfite, purchased from Difco Laboratories, U.S.A.
7. Buffered Peptone Water, purchased from Difco Laboratories, U.S.A.
8. Eosin Methylene Blue (EMB), purchased from Difco Laboratories, U.S.A.
9. Hektoen Enteric Agar, purchased from Difco Laboratories, U.S.A.
10. Lactobacilli MRS agar, purchased from Difco Laboratories, U.S.A.
11. Lactobacilli MRS broth purchased from Difco Laboratories, U.S.A.
12. Lactose, purchased from Difco Laboratories, U.S.A.
13. Lysine Iron Agar (LIA), purchased from Difco Laboratories, U.S.A.
14. Mannitol, purchased from Difco Laboratories, U.S.A.
15. Mannitol Salt Agar (MSA), purchased from Difco Laboratories, U.S.A.
16. Mannitol-Egg Yolk-Polymyxin (MYP) Agar, purchased from Difco Laboratories, U.S.A.
17. Methyl Red-Voges Proskauer (MR-VP) Broth, purchased from Difco Laboratories, U.S.A.
18. PALCAM Medium Base Agar, purchased from Difco Laboratories, U.S.A.
19. PALCAM Antimicrobic supplement, purchased from Difco Laboratories, U.S.A.

20. Phenol red broth Base, purchased from Hi Media Laboratories Pvt., Ltd., Mumbai., India.
21. Simmons citrate agar, purchased from Difco Laboratories, U.S.A.
22. Tetrathionate Broth Base, purchased from Difco Laboratories, U.S.A.
23. Tryptic Soy Broth (TSB), purchased from Difco Laboratories, U.S.A.
24. Triple Sugar Iron (TSI) Agar, purchased from Difco Laboratories, U.S.A.
25. UVM Modified *Listeria* Enrichment Broth, purchased from Difco Laboratories, U.S.A.
26. Xylose Lysine Deoxycholate (XLD), purchased from from Oxoid Ltd., England.
27. Yeast extract, purchased from Difco Laboratories, U.S.A.

Chemical used in this research are listed as follows:

1. Bromocresol purple blue, purchased from Fluka, Sigma-Aldrich Co., Inc., USA.
2. Crystal violet, purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
3. Disodium hydrogen phosphate (Na_2HPO_4), purchased from Merck KGaA, Germany
4. Ethanol absolute, Analytical grade, ACS., purchased from Scharlau Chemie S.A., Spain.
5. Glacial hydrochloric acid (HCl) (A.R. grade), purchased from Merck KGaA, Germany.
6. Glycerol (A.R. grade), purchased from Difco Laboratories, U.S.A.
7. Hydrogen peroxide 30%, purchased from Merck KGaA, Germany.
8. Iodine crystals, purchased from Merck KGaA, Germany.
9. Phenol Red Broth Base, purchased from Difco Laboratories, U.S.A.
10. Phenolphthalein, purchased from Ajax Finechem Pty., Ltd., Australia.
11. Polymyxin B Sulfate, purchased from Sigma-Aldrich Co., Inc., USA
12. Potassium dihydrogen phosphate (KH_2PO_4), purchased from Merck KGaA, Germany.
13. Potassium iodide (KI), purchased from Merck KGaA, Germany.

14. Potassium tellurite, purchased from Merck KGaA, Germany.
15. Salfanin O, purchased from Merck KGaA, Germany.
16. Sodium chloride (NaCl) (A.R. grade), purchased from Merck KGaA, Germany.
17. Sodium hydroxide (NaOH), purchased from Merck KGaA, Germany

Material and chemical used in classification of LAB and confirmation of pediocin gene are listed as follows:

1. Agarose gel, purchased from BIO-RAD Laboratories, Inc., Spain.
2. API 50 CH, purchased from BioMérieux, France.
3. API 50 CHL, purchased from BioMérieux, France.
4. 2 mM dNTPs mixture, purchased from Fermentas International Inc., Canada.
5. Ethylenediaminetetraacetic Acid (EDTA), purchased from Sigma-Aldrich Co., Inc., Singapore.
6. Forward primer pedF and reverse primer pedR, purchased from First BASE Laboratories Sdn., Bhd., Selangor Darul Ehsan, Malaysia.
7. Forward primer 16F27 and reverse primer 16R1522, purchased from First BASE Laboratories Sdn., Bhd., Selangor Darul Ehsan, Malaysia
8. Genome DNA Simax Kit, purchased from Beijing SBS Genetech Co., Ltd., China.
9. 100 bp Ladder Sharp DNA Marker, purchased from Fermentas International Inc., Canada.
10. 1 kb Ladder Sharp DNA Marker, purchased from Fermentas International Inc., Canada.
11. 6X Loading dye solution, purchased from Fermentas International Inc., Canada.
12. Lysozyme, purchased from Sigma-Aldrich Co., Inc., Singapore.
13. 25 mM Magnesium chloride ($MgCl_2$), purchased from Fermentas International Inc., Canada.
14. Proteinase K, purchased from Fermentas International Inc., Canada.
15. QIAGEN Plasmid Miniprep Kit (50), QIAGEN Sciences, Maryland, USA.

16. Sodium dodexyl sulfate (SDS), purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
17. Taq DNA polymerase, purchased from Fermentas International Inc., Canada.
18. 10X Taq buffer, purchased from Fermentas International Inc., Canada.
19. Trizma base, minimum 99.9% titration, purchased from Sigma-Aldrich Co., Inc., Singapore.

3.2 Instruments

1. Autoclave (Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan)
2. Cold room (Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K)
3. 4-Digit precision weighting balance (Model AG 204, Mettler Toledo, Switzerland)
4. DNA thermo cycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan)
5. Electrophoresis chamber set (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland)
6. Gel Documentation system (Bio-Rad Laboratories Gel Doc ^{TM} XR, California, U.S.A.)
7. High Speed Refrigerated Centrifuge (Beckman Coulter ^{TM} Avanti J-30I, Palo Alto, California, U.S.A.)
8. Hot air oven (Model UC 30, Memmert GmbH and Co. KG., Western Germany)
9. Hot plate stirrer (Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
10. Incubator (Model 800, Memmert GmbH and Co. KG., Western Germany)
11. Incubator shaker (Model SK-737, Amerex Instruments, Inc., USA)
12. Kubota Refrigerated Microcentrifuge 6500 (Kubota Corporation, Tokyo, Japan)
13. Laminar flow 'clean' (Model V6, Lab Service Ltd., Part)
14. Microscope (Model CH 30RF200, Olympus Optical Co., Ltd., Japan)
15. Microwave (Model 000502174, Thai Cityelectric Co. Ltd.,Thailand)
16. Orbital shaker (Innova Model, New Brunswick C0., Inc., USA)
17. pH meter (Mettler-Toledo International Inc., New York, U.S.A.)

18. Spectrophotometer (Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, USA)
19. Stomacher (Masticator, BEC-Thai, Thailand)
20. Stomacher bag (Seward Limited, UK)
21. UV-VIS spectrometer model V-530 (PC) (PerkinElmer instruments Lambda 25, Massachusetts, U.S.A.)
22. Vortex mixer (Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, USA)
23. Water bath (Model WB14, BEC-Thai Bangkok Equipment & Chemical Co., Ltd., Thailand)

3.3 Experimental Procedures

3.3.1 Isolation of Lactic acid bacteria (LAB) from nham

3.3.1.1 Nham sample collection

Twenty samples of nham from different lot of preparation kindly provided from Suddhiluck Innofood Co.,Ltd were used as resources for bacteriocin-producing LAB isolation. Before the experiment, all of nham samples were kept in cleaned container at 4°C.

3.3.1.2 Isolation of acid–producing bacteria

25 g of sample with 225 ml phosphate buffer pH 7.0 (appendix B) was homogenized in a stomacher bag (Seward Limited, UK) by stomacher (Masticator, BEC-Thai, Thailand) and ten-fold serially diluted was prepared in 0.85% normal saline solution (appendix B). Serial dilutions from each collected samples were plated on MRS agar supplemented with 0.004% bromocresol purple (appendix A) and incubated at 37°C for 48 hours under anaerobic conditions. Colonies producing yellow zones were

randomly picked from MRS agar plate (appendix A) and further purified by twice streaking on MRS agar. All of isolates were stabbed in MRS agar tall and kept at 4°C for use in the further experiments. The isolates were subcultured every 2 weeks.

3.3.1.3 Isolation of Lactic acid bacteria (LAB)

Pure culture of acid–producing isolates from 3.3.1.2 were determined for pre-identification of LAB based on morphological characteristics, Gram stain, catalase test and motility test (appendix C). Only those isolates which were cocci or rod shaped, Gram-positive, catalase-negative, and non-motile were selected for further studies and kept in MRS broth supplemented with 20% glycerol at -20°C.

3.3.2 Isolation of bacteriocin-producing LAB

LAB from 3.3.1.3 were tested for antimicrobial activity by using an agar spotted test and agar well diffusion method.

For agar spotted test, the procedure was imitated from Pongtep (2003). Isolated colonies were spotted on MRS agar and incubated at 37°C for 24 hours under anaerobic condition. Then, plates were overlaid with 0.75% TSAYE agar (appendix A) seeded with 10^7 $CFUml^{-1}$ of *Staphylococcus aureus* ATCC 25923 as a first indicator strain and incubated at 37°C for 24 hours under anaerobic conditions. Colonies producing inhibition zone were selected for studies with other indicator strains including *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311, *Listeria monocytogenes* DMST 17303 and *Bacillus cereus* ATCC 1729. All indicator strains obtained from Department of Microbiology, Faculty of Science, Chulalongkorn University. Colonies producing inhibition zone were confirmed with agar diffusion method.

For agar well diffusion method, the selected isolates were cultured in MRS broth (appendix A) at 37°C for 24 hours under anaerobic conditions. The supernatants obtained by centrifugation (Kubota Corporation, Tokyo, Japan) at 11,086 g 4°C for 20

minutes, then adjusted to pH 6.5 by 1 M NaOH (appendix B), and filtered through nylon filter membrane 0.45μ were prepared for next test. The procedure of agar well diffusion method was modified from that of Dawson et al. (2003) as shown in Figure 3.1

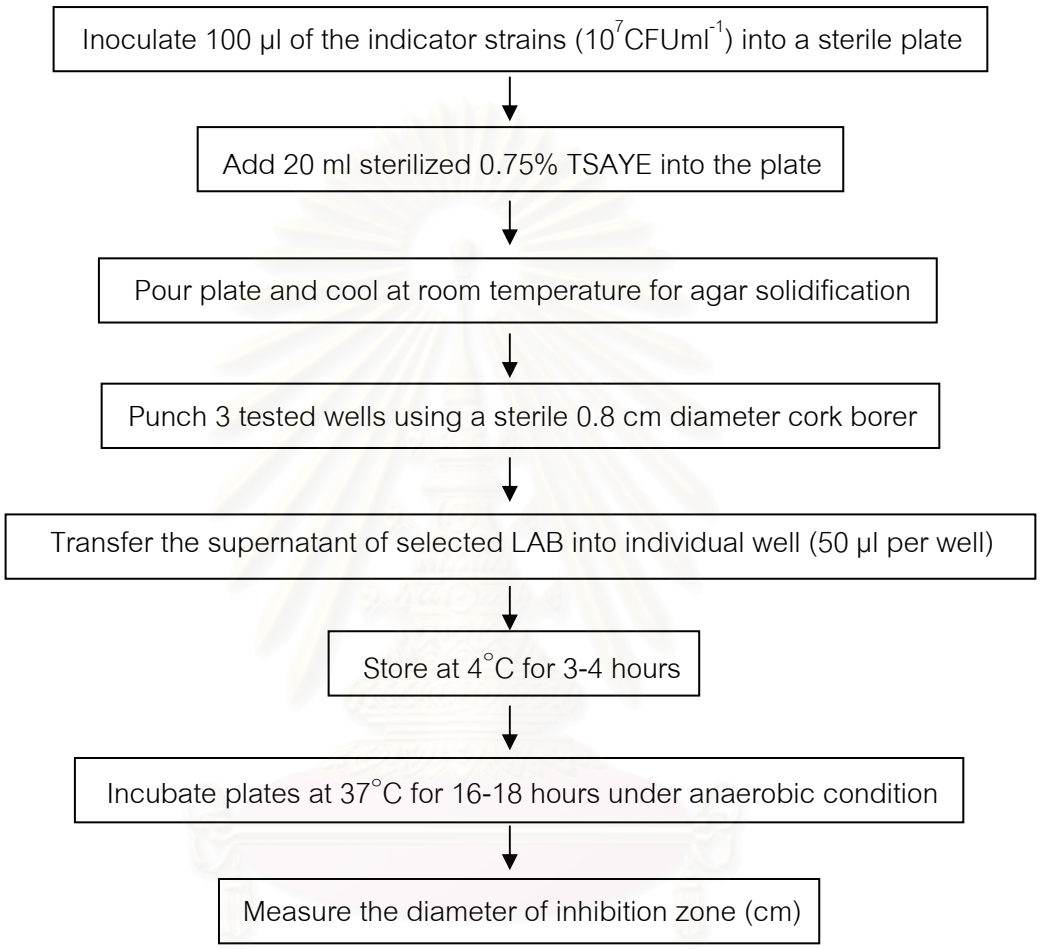


Figure 3.1 Procedure of agar well diffusion technique

3.3.3. Identification of bacteriocin-producing LAB

3.3.3.1 Genus Identification

Only the isolates, which had stable bacteriocin-producing activity were identified based on carbohydrates fermentation patterns of API 50 CHL identification kit (BioMérieux, France) (appendix C). Production of gas from glucose and growth at 10°C,

45°C, pH 4.4 and pH 9.6 were determined. In addition, salt tolerance was investigated by using 6.5% and 18% NaCl in MRS broth (appendix C). The selected strains were identified by following Bergey's Manual of Determinative Bacteriology.

3.3.3.2 Species Identification

16S rDNA analysis was used for the identification of LAB. The selected strains were cultured in 5 ml MRS broth and incubated at 37°C for 24 hours under anaerobic condition. Genomic DNA was extracted following the manufacturer's instructions of Genome DNA Simax Kit (Beijing SBS Genetech Co., Ltd., China). Genomic DNA was used as template to amplify 16S rDNA gene by the polymerase chain reaction method (PCR). PCR analysis followed procedure described by Horn et al. (1991) with some modifications.

PCR reagents mixture used for the amplification of 16S rDNA gene consisted of:

- 10X Taq buffer	5	µl
- 2 mM dNTPs mixture	5	µl
- 25 mM $MgCl_2$	3	µl
- Forward primer 16F27	2.5	µl
- Reverse primer 16R1522	2.5	µl
- Taq DNA polymerase	0.5	µl
- Distilled water	30.5	µl
- DNA template	1	µl

Universal primers were used following Bayane et al. (2006) that consisted of:

16F27 primer 5'-AGA GTT TGA TCC TGG CTC AG-3'

16R1522 primer 5'-AAG GAG GTG ATC CAG CCG CA-3'

PCR conditions were programmed as followed by 35 cycles of:

- Initial denaturation	94°C	1 minute
- Denaturation	94°C	1 minute
- Annealing	55°C	1 minute
- Extension	72°C	2 minutes
- Final extension	72°C	7 minutes

PCR amplification was carried out in a 50 µl mixture in a DNA thermo cycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan). PCR products were visualized by using gel electrophoresis (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland). When gel electrophoresis finished, agarose gel was stained with ethidium bromide and observed under UV light (Bio-Rad Laboratories Gel Doc ™ XR, California, U.S.A).

Sequencing of 16S rDNA gene was commercially serviced by Macrogen Inc. co. Ltd. (Seoul, Korea). The obtained sequences were blasted and compared alignment with the database of The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

3.3.4 Inhibitory efficiency of LAB on pathogenic bacteria in MRS broth

3.3.4.1 Growth profile and specific growth rate of pathogenic bacteria

Growth of pathogenic bacteria that used in this experiment including *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* DMST 17303, *Bacillus cereus* ATCC 1729, *Salmonella* Typhimurium ATCC 13311 and *Escherichia coli* ATCC 25922 were studied. Single colony of each pure strain was inoculated in 5 ml Tryptic soy broth (TSB) (appendix A) and incubated at 37°C for 1 hour under orbital shaking 200 rpm for preparing the inoculum. This experiment was divided into five groups include:

Group 1 : 50 ml TSB in kettle flask for blank setting

Group 2-4 : 50 ml TSB in kettle flask inoculated with 500 µl of inoculum for measuring the optical density

Group 5 : 50 ml TSB in kettle flask inoculated with 500 µl of inoculum for measuring bacteria count

All experimental groups excepted group 1 were incubated at 37°C under orbital shaking 200 rpm. Growth of bacteria was followed by measuring the optical density at 600 nm (OD_{600}) every 1 hour with spectrophotometer Genesys 20 Model 4001/4, ThermoSpectronic, and Rochester., New York, USA) until constant absorbance was obtained. The result was calculated for specific growth rate (µ, hr^{-1}) and doubling time (hour). Calculation was shown in appendix E and graph was plotted between OD_{600} (nm) and time (hour). Viable bacteria count was determined by spreading 100 µl of cell suspension on Tryptic Soy Agar plate (TSA) (appendix A) and incubated at 37°C for 24 hours under aerobic condition. Viable bacteria count was shown in term of log $CFUml^{-1}$. The experiment was conducted in triplicate.

3.3.4.2 Growth profile and specific growth rate of LAB

Procedure of growth experiment of LAB was similar to the pathogenic bacteria. Firstly, growth of selected LAB were studied and measured only at 24 hours of incubation time for preparing the approximately inoculum using in the study of the inhibitory efficiency of LAB on pathogenic bacteria in MRS broth. After the highest inhibitory efficiency of LAB was selected, growth of the best selected LAB was studied.

Single colony of LAB was inoculated in 5 ml MRS broth and incubated at 37°C for 1 hour under anaerobic condition for preparing the inoculum. This experiment was divided into five groups include:

Group 1 : 50 ml MRS broth in kettle flask for blank setting

Group 2-4 : 50 ml MRS broth in kettle flask inoculated with 500 µl of inoculum for measuring the optical density

Group 5 : 50 ml MRS broth in kettle flask inoculated with 500 µl of inoculum for measuring LAB count

After adding inoculum to experimental groups 2, 3, 4 and 5, all experimental groups excepted group 1 were incubated at 37°C for 24 hours under anaerobic condition. The optical density at 600 nm (OD_{600}) was measured every one or two hours until constant absorbance was obtained. The result was calculated for specific growth rate (µ, hr^{-1}) and doubling time (hour). Calculation was shown in appendix E and graph was plotted between OD_{600} (nm) and time (hour). LAB count was determined by spreading 100 µl of cell suspension on MRS agar plate and incubated at 37°C for 24 hours under anaerobic condition. LAB count was shown in term of log $CFUml^{-1}$. The experiment was conducted in triplicate.

3.3.4.3 Preparation bacteria for the experiment

Inhibitory effect of selected LAB against pathogenic bacteria as described previously in 3.3.4.1 was done by co-culturing pathogenic bacteria with LAB in MRS broth. The initial cell concentration of LAB and pathogenic bacteria using in this experiment were equaled to 10^6 and 10^4 $CFUml^{-1}$, respectively. The experimental groups were divided into three groups include:

control A : 99 ml MRS broth inoculated with only 1 ml LAB

control B : 99 ml MRS broth inoculated with only 1 ml pathogenic bacteria

co-cuture : 98 ml MRS broth inoculated with 1 ml LAB and 1 ml pathogenic bacteria

Selected LAB was cultured in MRS broth and proceeded through the procedure as shown in Figure 3.2

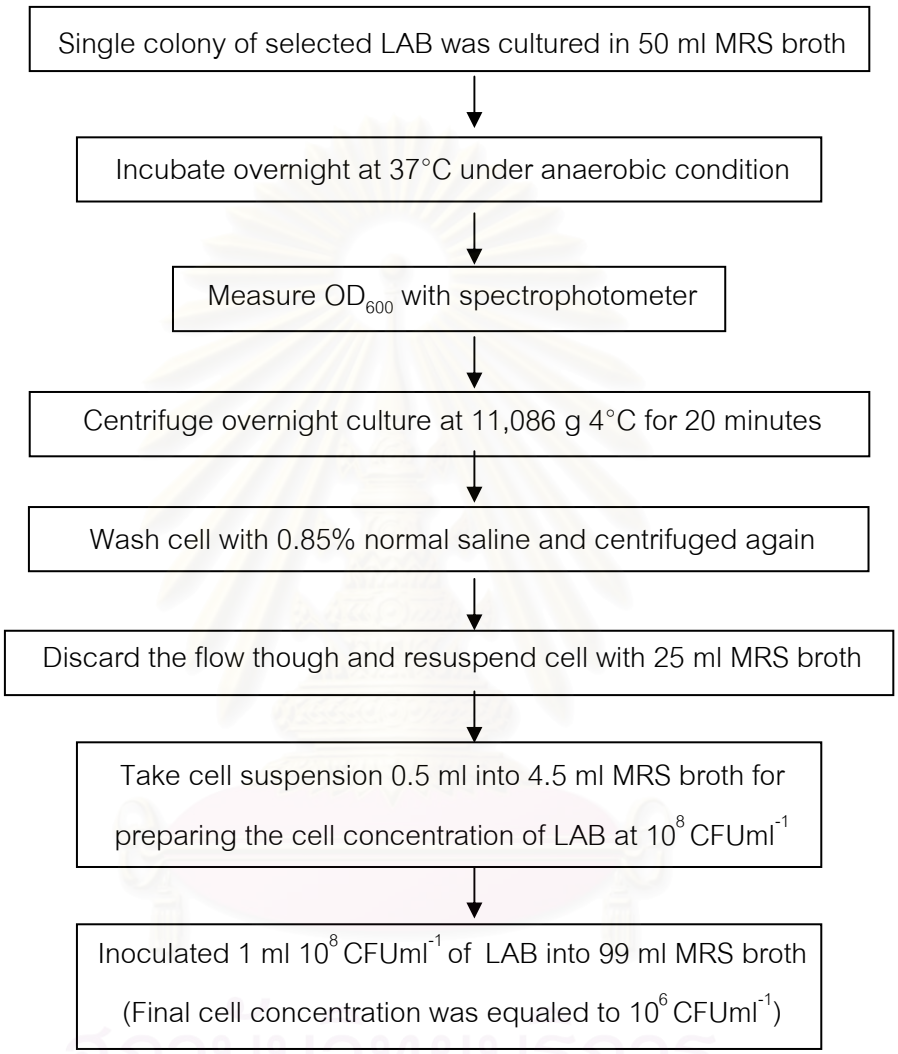


Figure 3.2 Preparation of LAB inoculum at desired level for studying the inhibitory efficiency of LAB on pathogenic bacteria in MRS broth

Pathogenic bacteria was cultured in TSB and proceeded through the procedure as shown in Figure 3.3

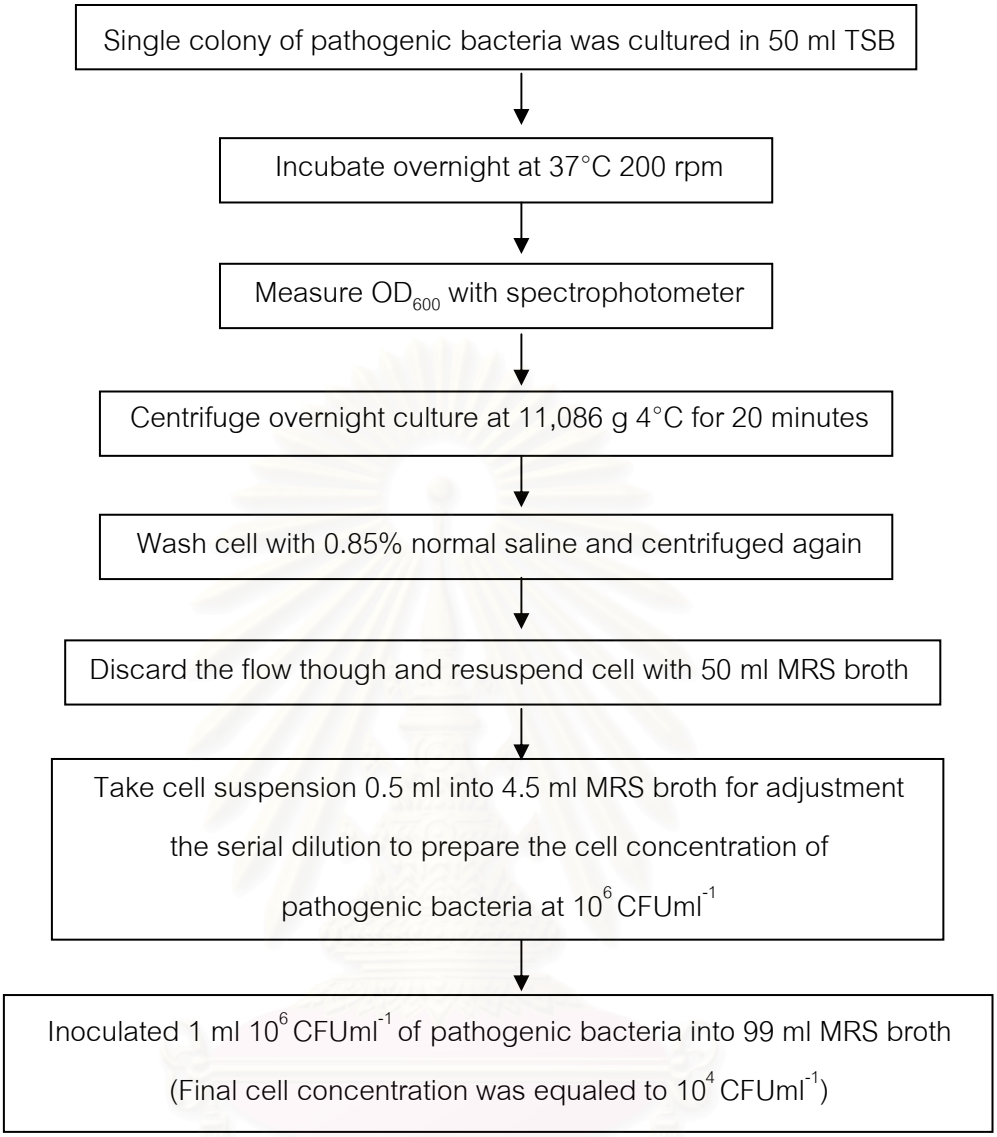


Figure 3.3 Preparation of pathogenic bacteria inoculum at desired level for studying the inhibitory efficiency of LAB on pathogenic bacteria in MRS broth

All experimental groups were incubated at 37°C for 48 hours. Sampling was done at 0, 4, 8, 12, 16, 24 and 48 hours for viable bacteria counts, pH measurement and bacteriocin detection.

3.3.4.4 Determination of bacteria, pH and bacteriocin activity in MRS broth during incubation time

3.3.4.4.1 Determination of viable bacteria counts

Viable bacteria counts were determined by aseptically sampling 0.5 ml MRS broth from all experimental groups into 4.5 ml 0.85% NaCl. Appropriate decimal dilutions of the samples were prepared using the same diluent. Aliquot of 0.1 ml of each dilution was plated in triplicate on different media. For LAB count, MRS agar was used and incubated at 37°C for 24 hours under anaerobic condition. For viable bacteria counts, TSA was used and incubated at 37°C for 24 hours under aerobic condition.

LAB and viable bacteria counts were shown in term of log $CFUml^{-1}$ and graph was plotted the relationship between LAB or viable bacteria counts (log $CFUml^{-1}$) and time (hour).

3.3.4.4.2 Confirmation of pathogenic bacteria

Pathogenic bacteria were confirmed on appropiated selective media including Mannitol Salt Agar (MSA) (appendix A) for *S. aureus*, PALCAM medium base agar (appendix A) for *L. monocytogenes*, Eosin Methylene Blue agar (EMB) (appendix A) for *E. coli* and Xylose Lysine Deoxycholate agar (XLD) (appendix A) for *S.* Typhimurium. All microorganisms were streaked and incubated at 37°C for 24 hours under aerobic condition.

3.3.4.4.3 Determination of pH and bacteriocin activity

MRS broth from co-culture group was taken approximately 5 ml and centrifuged at 11,086 g 4°C for 20 minutes. Cell-free supernatant was measured by pH meter (Mettler-Toledo International Inc., New York, U.S.A.). The relationship between pH and time (hour) were plotted as a graph.

After pH measurement, bacteriocin production was tested. Supernatant was adjusted to pH 6.5 with 0.1 N NaOH, filtered through nylon membrane filter 0.45 µ. Bacteriocin was detected using agar diffusion method as described previously in 3.3.2. *S. aureus* was used as an indicator strain. LAB which had the highest inhibitory effect against pathogenic bacteria in MRS broth was selected for further experiments.

3.3.5 Evaluation of environmental conditions for production of bacteriocin

The temperature, pH, NaCl concentration and incubation time were tested to elucidate the appropriate conditions for exhibiting the maximum bacteriocin production of LAB (Pongtep, 2003).

3.3.5.1 Preparation of the inoculum

Single colony of LAB from 3.3.4 was cultured in 5 ml MRS broth and incubated at 37°C for 18 hours under anaerobic condition for preparing the inoculum.

3.3.5.2 Effect of temperature on the bacteriocin production

0.1 ml of inoculum from 3.3.5.1 was added into 10 ml MRS broth and separately incubated at different temperature including 25°C, 30°C, 35°C, 37°C, 40°C, and 45°C for 24 hours under anaerobic condition. Growth of LAB was followed by measuring the optical density at 600 nm (OD_{600}) with spectrophotometer. In addition, pH and bacteriocin activity were measured.

pH measurement was done by centrifugation of cultured media at 11,086 g 4°C for 20 minutes. Cell-free supernatant was measured by pH meter.

After pH measurement, bacteriocin activity was done. Procedure of bacteriocin activity measurement was modified from Mayr-Harting et al. (1972). Supernatant was adjusted to pH 6.5 with 0.1 N NaOH, filtered through nylon membrane filter 0.45 µ. Two-fold serial dilutions of the cell-free supernatant were made in sterile

distilled water. The activities of 10 µl cell-diluted samples were determined by agar diffusion method as described previously in 3.3.2. *S. aureus* was used as an indicator strain. The titer of bacteriocin activity was expressed in terms of arbitrary units per ml ($AUml^{-1}$). The arbitrary units was determined as the reciprocal of the highest dilution showing inhibition of the indicator strain (Appendix E).

3.3.5.3 Effect of pH on the bacteriocin production

0.1 ml of inoculum from 3.3.5.1 was added into 10 ml MRS broth adjusting for different pH value including 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 9.5 and incubated at 37°C for 24 hours under anaerobic condition. Growth measurement, pH and bacteriocin activity were measured using similar procedure as previous description in 3.3.5.2.

3.3.5.4 Effect of NaCl on the bacteriocin production

0.1 ml of inoculum from 3.3.5.1 was added into 10 ml MRS broth preparing in different NaCl concentration including 0, 1, 3, 5, 7, 9, 11, 13 and 15 %w/v and incubated at 37°C for 24 hours under anaerobic condition. Growth measurement, pH and bacteriocin activity were measured using similar procedure as previous description in 3.3.5.2.

3.3.5.5 Effect of incubation time on the bacteriocin production

0.1 ml of inoculum from 3.3.5.1 was added into 10 ml MRS broth and incubated at different time including 12, 24 and 36 hours at 37°C under anaerobic condition. Growth measurement, pH and bacteriocin activity were measured using similar procedure as previous description in 3.3.5.2.

3.3.6 Inhibitory efficiency of LAB on pathogenic bacteria in nham as food model

3.3.6.1 Preparation of LAB as starter culture

LAB was cultured in MRS broth and proceeded through the procedure as shown in Figure 3.4

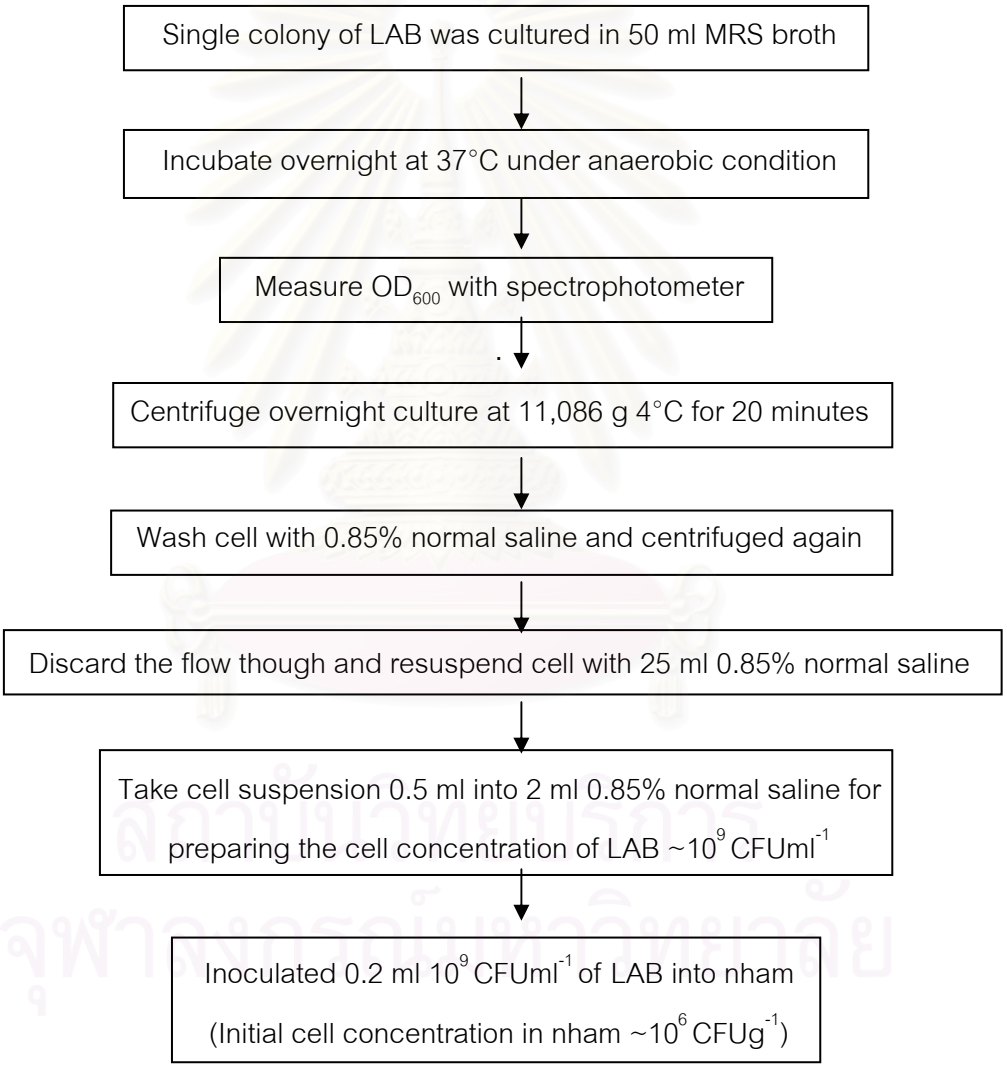


Figure 3.4 Preparation of LAB using as starter culture in nham

3.3.6.2 Preparation of pathogenic bacteria

Pathogenic bacteria were used in this experiment including *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S.* Typhimurium ATCC 13311, *L. monocytogenes* DMST 17303 and *B. cereus* ATCC 1729 cultured in TSB and proceeded through the procedure as shown in Figure 3.5

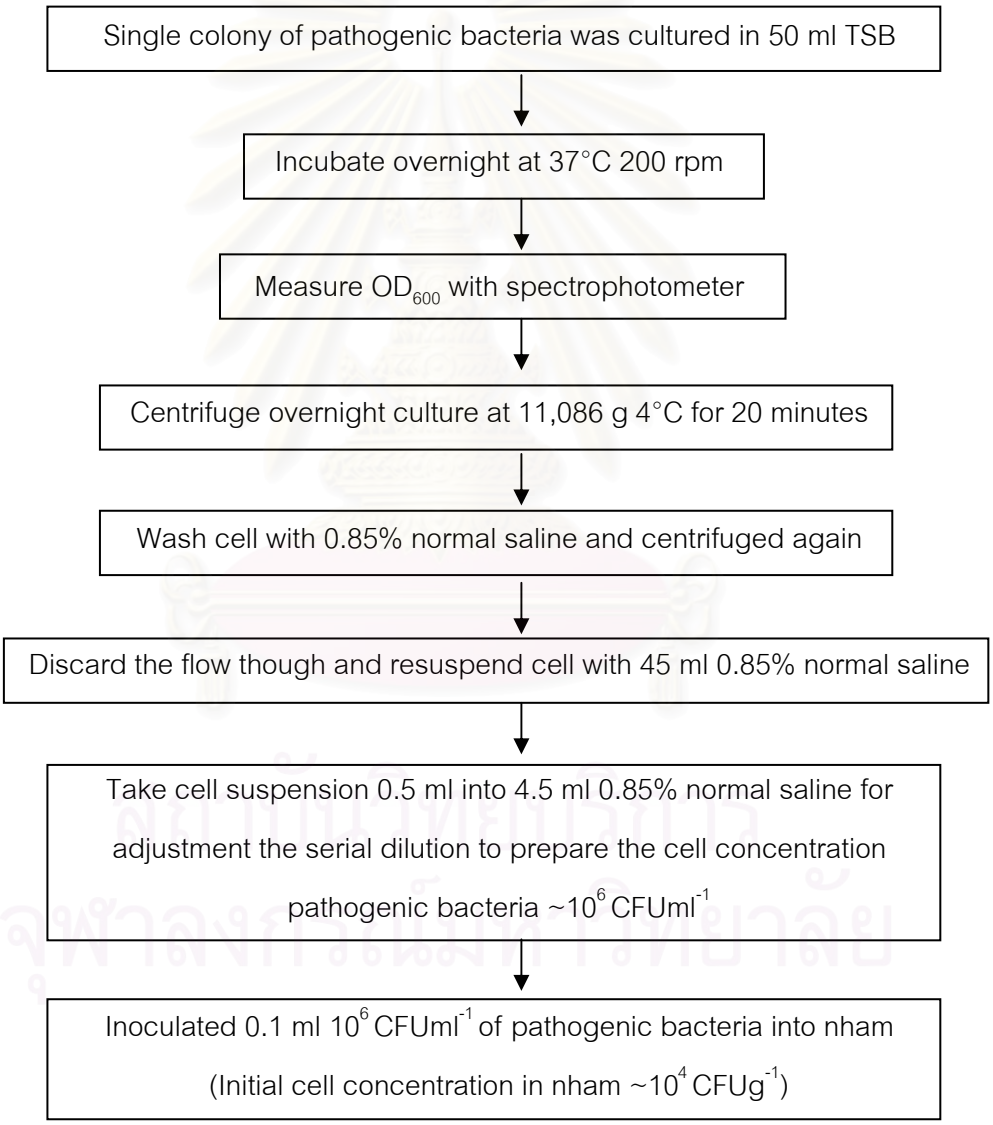


Figure 3.5 Preparation of pathogenic bacteria for inoculation to nham

3.3.6.3 Nham preparation

Nham was prepared and modified from Nettasud (2006) by combining a mixture of minced pork 40%, cooked rice 3.5%, garlic 0.2%, sugar 0.2%, salt 0.2% and chili 2%. The mixture was thoroughly mixed, then tightly packaged in plastic bags (approximately 20 g each) and tightly sealed with rubber band. The experimental of nham were divided into 4 batchs including

Batch 1: NNF (naturally fermentation of nham as a control)

Batch 2: NS (nham inoculated with 10^6 $CFUg^{-1}$ of LAB)

Batch 3: NP (nham inoculated with 10^4 $CFUg^{-1}$ of pathogenic bacteria)

Batch 4: NSP (nham inoculated with 10^6 $CFUg^{-1}$ of LAB and 10^4 $CFUg^{-1}$ pathogenic bacteria)

Nham were incubated at 30°C for 7 days and samples were taken at 0, 24, 72, 120 and 168 hours for total LAB count, pathogenic bacteria count, total viable bacteria counts in nham sample, pH measurement and total acidity (%w/v) analysis.

3.3.6.4 Microbiological determination

3.3.6.4.1 Determination of total LAB and total viable bacteria counts

10 g of nham samples were aseptically transferred to a sterile stomacher bag containing with 90 ml of buffered peptone water (BPW) (appendix B) and pummelled for 1 minute in stomacher. Appropriate ten-fold serially dilutions of the homogenized samples were prepared in 0.85% normal saline solution. Serial dilutions from each sample were plated in triplicate on different media. For total LAB count, aliquot of diluted sample was plated on MRS agar and incubated at 37°C for 24 hours under anaerobic condition. For total viable bacteria counts, aliquot of diluted sample was plated on TSA and incubated at 37°C for 24 hours under aerobic condition.

3.3.6.4.2 Determination of *S. aureus*

The procedure for isolation and identification of *S. aureus* in nham sample was modified from Petchsing and Woodburn (1990) that shown in Figure 3.6

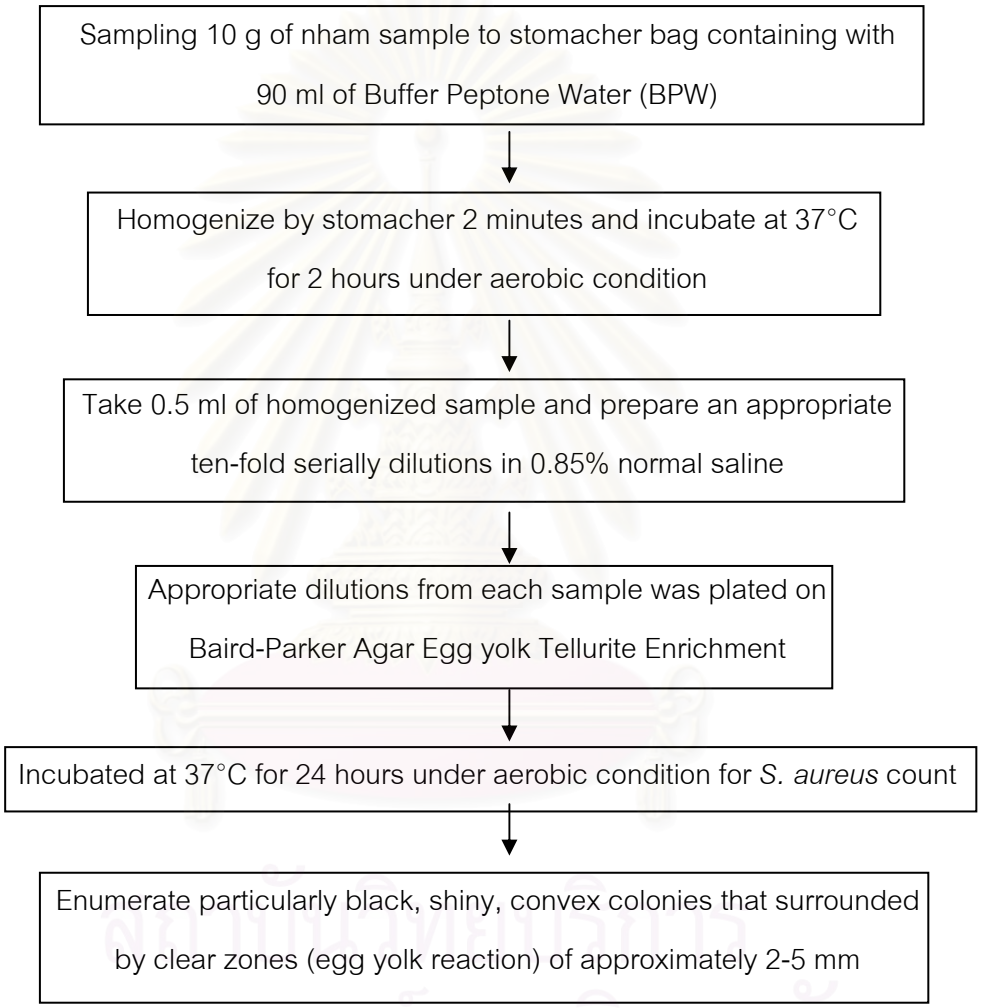


Figure 3.6 Procedure for isolation and enumeration of *S. aureus* in nham

3.3.6.4.3 Determination of *L. monocytogenes*

The procedure for isolation and identification of *L. monocytogenes* in nham sample was modified from Hitchins (2000) that shown in Figure 3.7

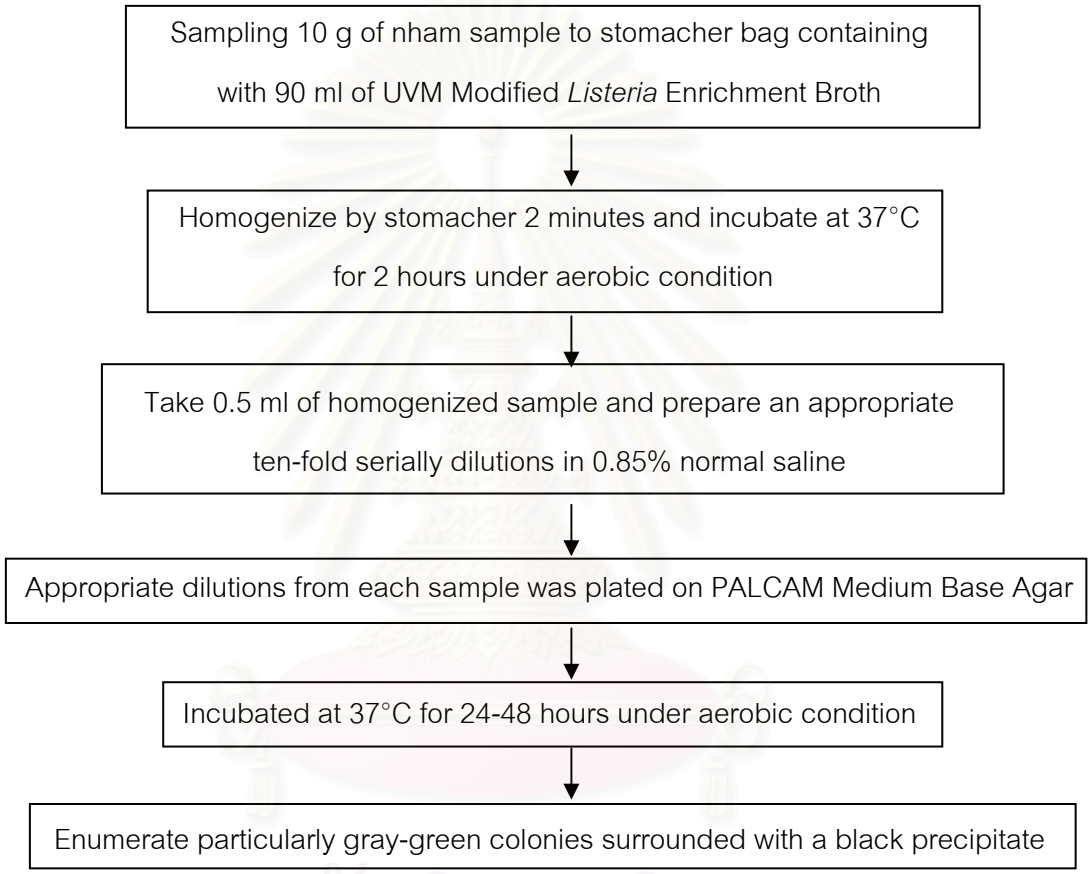


Figure 3.7 Procedure for isolation and enumeration of *L. monocytogenes* in nham

3.3.6.4.4 Determination of *B. cereus*

The procedure for isolation and identification of *B. cereus* in nham sample was modified from Peng et al. (2001) that shown in Figure 3.8

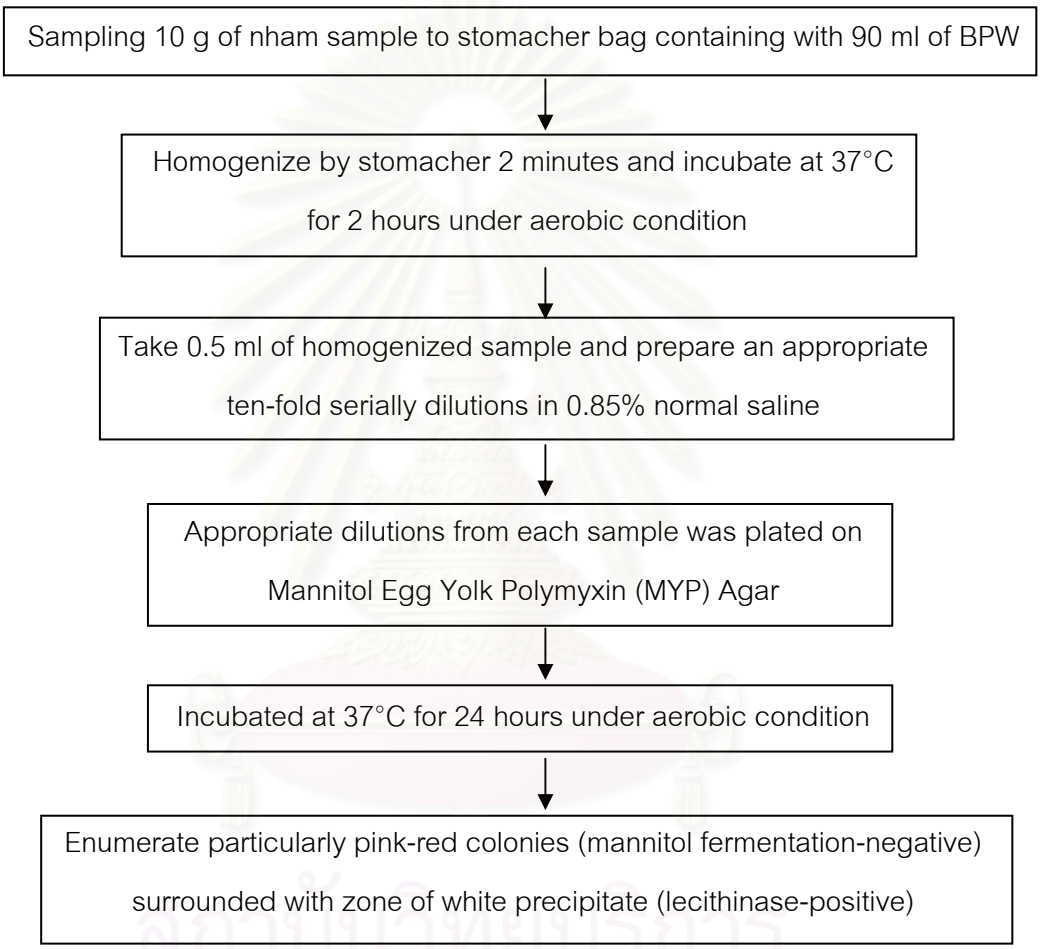


Figure 3.8 Procedure for isolation and enumeration of *B. cereus* in nham

3.3.6.4.5 Determination of *E. coli*

The procedure for isolation and identification of *E. coli* in nham sample was modified from Hitchins et al. (1998) that shown in Figure 3.9

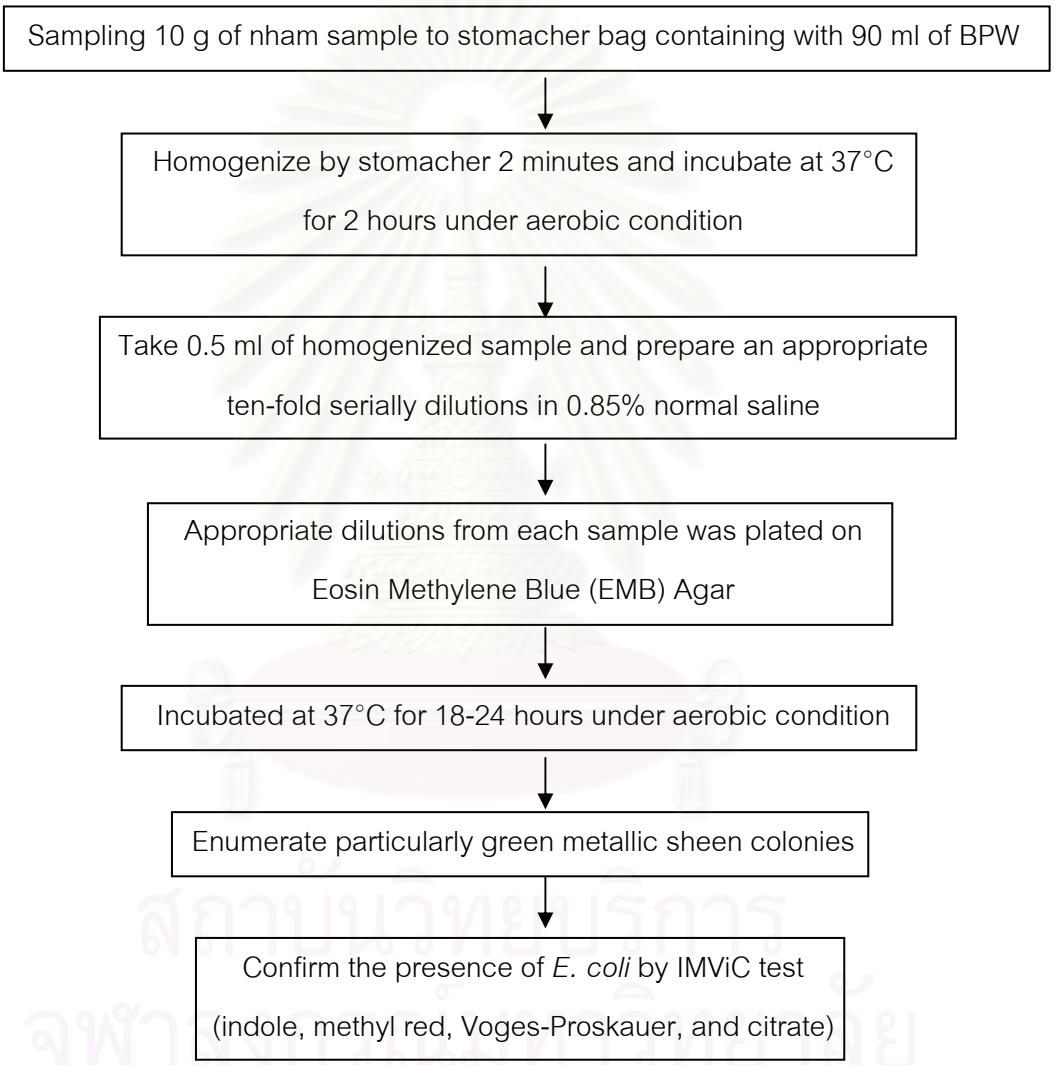


Figure 3.9 Procedure for isolation and enumeration of *E. coli* in nham

3.3.6.4.6 Determination of *S.* Typhimurium

The procedure for isolation and identification of *S.* Typhimurium in nham sample was modified from Andrews and Hammack (1998) that shown in Figure 3.10

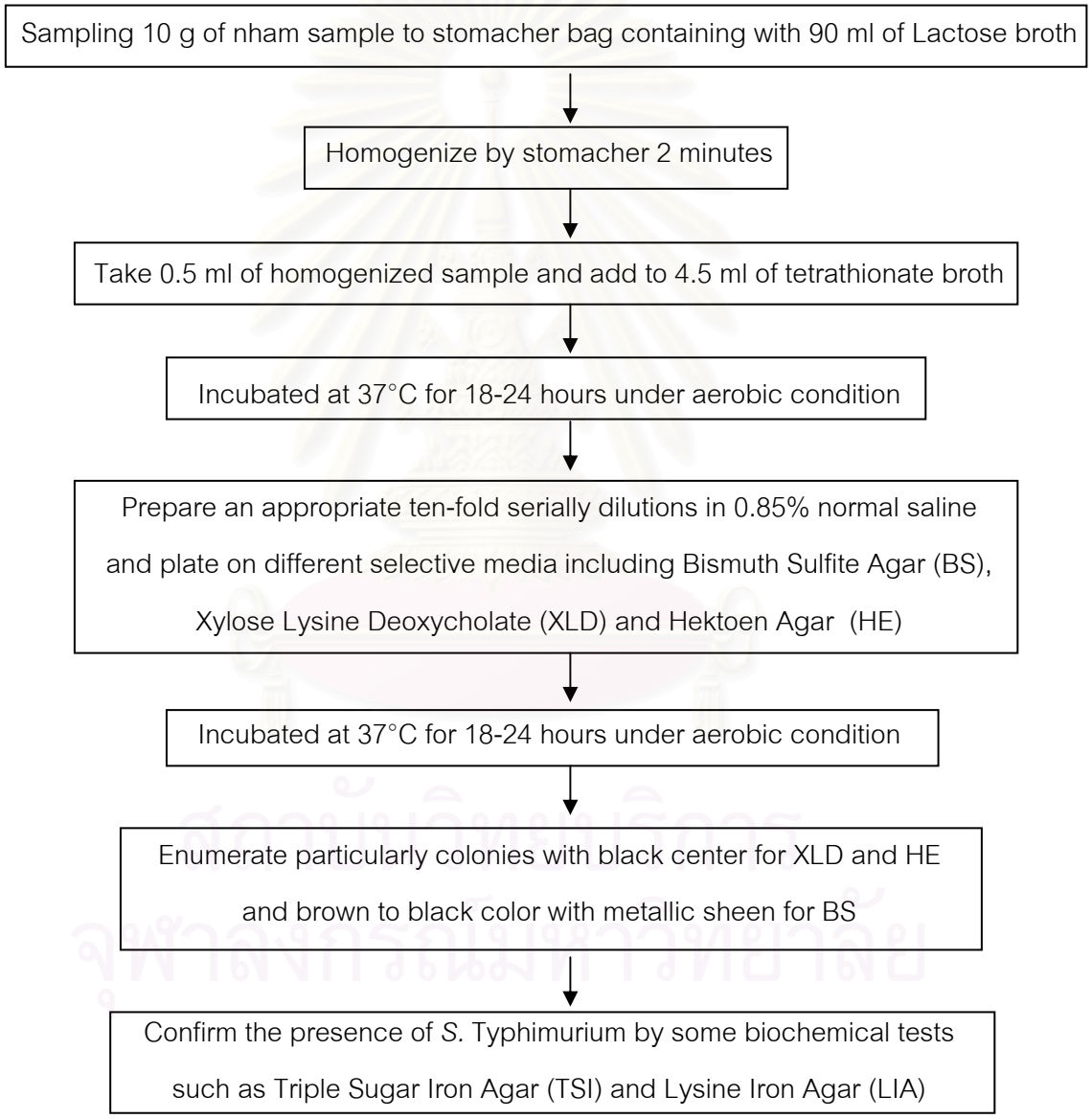


Figure 3.10 Procedure for isolation and enumeration of *S.* Typhimurium in nham

3.3.6.5 Determination of pH and total acidity (%w/v)

pH and %relative total acidity (%w/v) of nham samples were determined by following the procedure of Benjakul et al. (1997) and AOAC (2000).

For pH measurement, 5 g of nham sample was added to bag containing 40 ml of CO_2-free distilled water and homogenized for 1 minute by using a stomacher. The pH of homogenate was directly measured by pH meter. Relationship between pH value and time (hour) were plotted as a graph.

After pH measurement, the homogenate was centrifuged at 3,000 g for 15 minutes at room temperature. Supernatant was filtered through a Whatman No.4 filter paper. 20 g of the filtrate was titrated with the standardized 0.1 M NaOH using 1%w/v phenolphthalein as an indicator. Calculation of %total acidity based on lactic acid production was expressed as percentage (%w/v) that shown in appendix E. Total acid was shown in term of relative of total acid increase and plotted versus time (hour).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of Lactic acid bacteria (LAB) from nham

On the screening of Lactic acid bacteria (LAB), sources of isolation are very important. LAB are ubiquitous found in many food fermentation including dairy, meat, vegetable and bakery products (Marrug, 1991). Especially in meat, LAB constitute a part of initial microflora which develop easily after meat is passed fermentation process. In addition, the important roles of LAB in fermented food are caused uniqueness in flavour and texture changes together with a preservative effect. Therefore, nham, a Thai tradition fermented pork was used as a source to isolate LAB strains in this study because raw minced pork was the major ingredient which comprised in nham production and the physico-chemical and biochemical changes in nham were associated with indigenous LAB flora.

In this study, a total of 107 LAB isolated from 20 samples of nham, were collected from Suddhiluck Innofood Co.,Ltd. They were forty-two rod-shaped and sixty-five cocci-shaped bacteria. All LAB isolates were Gram-positive, catalase-negative, and non-motile as shown in Table E1 (appendix F).

4.2 Bacteriocin-producing LAB from nham

The purpose of this study associated with the application of bacteriocin-producing LAB against certain pathogenic bacteria for using as starter culture in nham preparation. To achieve this propose, 107 LAB isolates were tested for antimicrobial activity by using agar spotted test and agar well diffusion method. Both of two methods were used *S. aureus* ATCC 25923 as a first indicator strain for the initial screening because meat and meat products are commonly involved with staphylococcal food poisoning (Varnam, 1995). In general, *S. aureus* are often presented in fresh tissues that

related to human handling, meat handling surfaces and careless of slaughter. Many strains of *S. aureus* are able to produce enterotoxins that cause food poisoning (Robert, 1982). The presence of *S. aureus* in food is caused a potential public health hazard. Therefore, LAB which had bactericidal effect on *S. aur*eus was the one among this research interests.

For spotted test method, 65 LAB isolates were found to inhibit *S. aureus* from the presence of inhibition zone surrounding LAB colony as shown in Table F2 (appendix F). Then, their bacteriocin-producing activity was tested with various indicator strains. There were LAB isolates 6, 5, 3 and 1 that found to exhibit inhibitory effect against with *B. cereus*, *L. monocytogenes*, *E. coli* and *S.* Typhimurium, respectively. After that agar well diffusion method were used for confirming bacteriocin production of 65 LAB isolates. Only 34 isolates could perform inhibitory effect against *S. aureus* as shown in Table 4.1. In contrast, they could not inhibit *B. cereus*, *L. monocytogenes* and Gram-negative bacteria.

Table 4.1 Bacteriocin-producing LAB against *S. aureus* isolated by agar well diffusion method

Isolate number	Inhibition zones (cm)
NSL8-1	1.97 ± 0.06
NSL11-1	2.00 ± 0.00
NSL14-1	1.67 ± 0.12
NSL15-1	1.60 ± 0.00
NSL16-1	1.70 ± 0.00
NSL20-1	1.57 ± 0.12
NSL21-1	1.60 ± 0.00
NSL22-1	1.60 ± 0.10
NSL26-1	1.50 ± 0.00

Isolate number	Inhibition zones (cm)
NSL33-1	1.40 ± 0.00
NSL39-1	1.63 ± 0.06
NSL1-2	1.10 ± 0.00
NSL4-2	1.40 + 0.00
NSL5-2	1.57 + 0.06
NSL7-2	1.53 + 0.06
NSL9-2	1.77 + 0.06
NSL10-2	1.43 + 0.06
NSL12-2	1.47 + 0.06
NSL13-2	1.77 + 0.06
NSL9-3	1.63 + 0.12
NSL20-3	1.73 + 0.21
NSL23-3	2.33 + 0.06
NSL25-3	1.67 + 0.12
NSL26-3	1.60 + 0.00
NSL27-3	1.87 + 0.12
NSL1-4	1.83 + 0.06
NSL2-4	1.50 + 0.00
NSL5-4	1.83 + 0.15
NSL6-4	1.67 + 0.06
NSL9-4	1.50 + 0.00
NSL16-4	1.53 + 0.06
NSL25-4	1.60 + 0.00
NSL30-4	1.80 + 0.10
NSL31-4	1.77 + 0.06

According to the results, bacteriocin-producing LAB isolates that gave a positive result on a spotted test method would give negative result on agar well diffusion method. For spotted test method, the inhibitory effects against indicator strains were caused not only from bacteriocin activity but also other antimicrobial substances such as organic acid. To eliminate the effect of acid production, cell-free supernatant obtained from cultured broth was adjusted to pH 6.5 and then tested by using agar well diffusion assay as shown in Figure 4.1. The advantage of this method was simple, saving time and low-cost. Limitation would occur from a low inhibition zone but could be improved by allowing some time for bacteriocin to diffuse into agar prior the incubation or increasing well size for more sample addition. On the other hand, the use of high concentrated-supernatant was a way to get rid of problem from this method (Lewus et al., 1991).

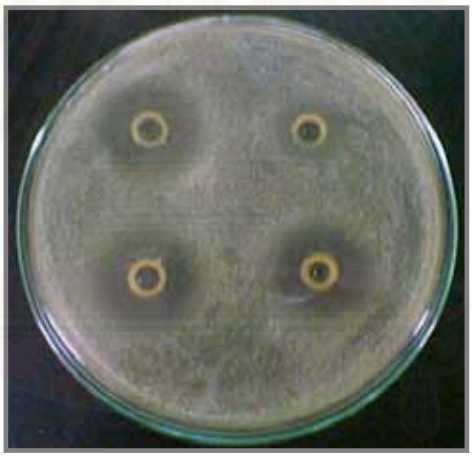


Figure 4.1 Agar well diffusion method

The target of bacteriocins is the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipids bilayer (Montville et al., 1995). Due to the protective barrier provided by the lipopolysaccharides (LPS) and glycerophospholipids of the outer membrane (OM), which covers the cytoplasmic membrane and peptidoglycan layer of Gram-negative cells (Belfiore et al., 2007). Therefore, bactericidal activity of bacteriocin is more effective against closely related species of producer and Gram-positive bacteria than Gram-negative bacteria.

However, mutant strains and protoplasts of Gram-negative bacteria can cause sensitive to bacteriocin action after exposure to sub-lethal stress such as heating, freezing, or thawing, which disrupt the outer membrane and allow the attachment of bacteriocin to the cytoplasmic membrane that increased sensitivity (Stevens et al,.1991).

4.3 Identification of bacteriocin-producing LAB

4.3.1 Genus Identification

Previously researches described that bacteriocin genes can be present on both of the bacterial chromosome and plasmids (Steen et al., 1991; Miller et al., 2005). If bacteriocins are plasmid-mediated proteins, plasmids could be lost from some cultures after many times of subculture. Therefore, in this study only strains which performed the stability of bacteriocin-producing activity were selected for identification.

Strain NSL13-2, NSL1-4 and NSL5-2 were stably produced bacteriocin and selected for identification based on carbohydrate fermentation patterns of API 50 CHL identification kit (BioMérieux, France). The results showed in Table 4.2. Carbohydrates fermentation using identification software in http://apiweb.biomerieux.com, NSL13-2, NSL1-4 and NSL5-2 were identified as *Pediococcus pentosaceus*, *Weissella confusa* and *Lactobacillus* sp., respectively. The identity percentage (%ID) of NSL13-2 was 99.9 % that offered an excellent identification profile. But the API identification profiles of NSL1-4 and NSL5-2 were doubtful and unacceptable, respectively. However, NSL13-2, NSL1-4 and NSL5-2 were finally identified by 16S rDNA analysis.

Moreover, production of gas from glucose and growth at 10°C, 45°C, pH 4.4 and pH 9.6 were determined. In addition, salt tolerance was investigated by using 6.5% and 18% NaCl in MRS broth. The results were shown in Table 4.3.

Table 4.2 Carbohydrates fermentation pattern of strains NSL13-2, NSL1-4 and NSL5-2 using API 50 CHL (BioMérieux, France)

Carbohydrate	Isolate number		
	NSL13-2	NSL1-4	NSL5-2
Control	-	-	-
Glycerol	-	-	+
Erythritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	+	+	-
Ribose	+	-	+
D-XyLose	-	+	-
L-XyLose	-	-	-
Adonitol	-	-	+
β-Methyl-D-Xyloside	-	-	-
Galactose	+	-	+
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Sorbose	-	-	-
Rhamnose	+	-	-
Dulcitol	-	-	-
Inositol	-	-	-
Mannitol	-	-	+
Sorbitol	-	-	+
α-Methyl-D-Mannoside	-	-	?
α-Methyl-D-Glucoside	-	-	?

Carbohydrate	Isolate number		
	NSL13-2	NSL1-4	NSL5-2
N-Acetyl-Glucosamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin	+	+	+
Salicin	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	-	-	+
Melibiose	+	-	+
Sucrose	+	+	+
Trehalose	+	-	+
Inulin	-	-	-
Melezitose	-	-	+
Raffinose	+	-	?
Starch	-	-	-
Glycogen	-	-	-
Xylitol	-	-	?
Gentiobiose	+	+	+
D-Turanose	-	-	-
D-Lyxose	-	-	-
D-Tagatose	+	-	+
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabitol	-	-	+
L-Arabitol	-	-	+

Carbohydrate	Isolate number		
	NSL13-2	NSL1-4	NSL5-2
Gluconate	-	-	+
2-Keto-Gluconate	-	-	?
5-Keto-Gluconate	-	-	-

+ = positive; - = negative; ? = doubtful

Table 4.3 The characteristics of strains NSL13-2, NSL1-4 and NSL5-2

Characteristics	Isolate number		
	NSL13-2	NSL1-4	NSL5-2
Cell morphology	Cocci	Coccobacilli	Cocci or oval
Gram stain	positive	positive	positive
Catalase test	negative	negative	negative
Gas production from glucose	NG	G	NG
Growth at temperature			
10°C	+	-	-
45°C	+	-	+
Growth at pH			
4.4	+	+	-
9.6	+	-	+
Growth at NaCl			
6.5%	+	+	+
18%	-	-	-

+ = growth; - = no gowth; NG = Not produce CO_2; G = produce CO_2

Colonial appearance on MRS agar and the characteristic under microscope of NSL13-2, NSL1-4 and NSL5-2 on MRS agar were shown in Figure 4.2, 4.3 and 4.4, respectively.

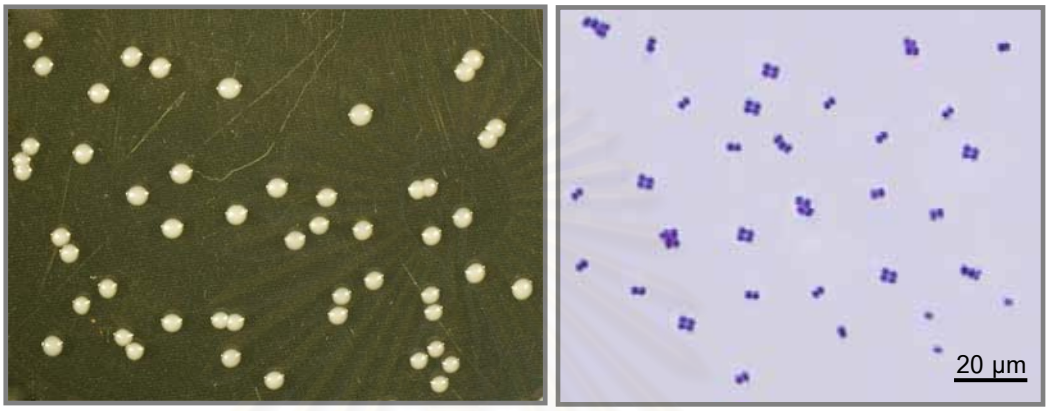


Figure 4.2 Colonial appearance on MRS agar and the characteristic under microscope of NSL13-2 (magnification 1,000x)

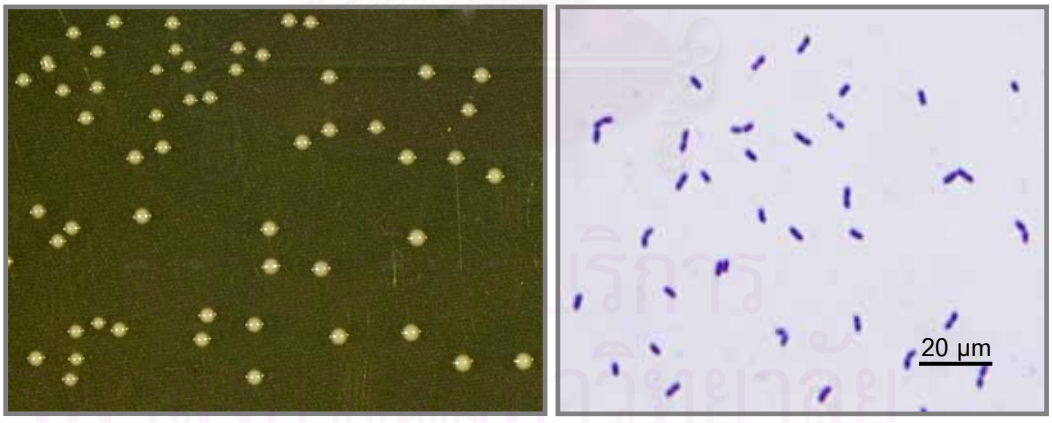


Figure 4.3 Colonial appearance on MRS agar and the characteristic under microscope of NSL1-4 (magnification 1,000x)

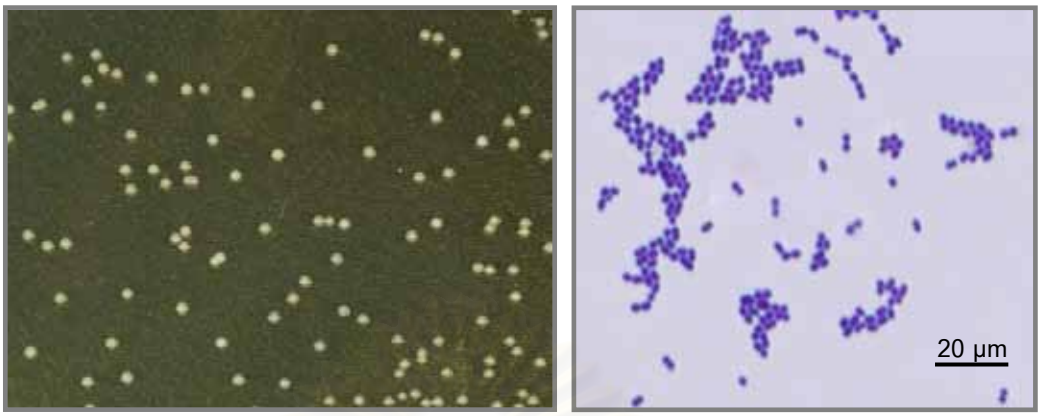


Figure 4.4 Colonial appearance on MRS agar and the characteristic under microscope of NSL5-2 (magnification 1,000x)

4.3.2 Species Identification

After API 50 CHL identification and some biochemical tests were done, genomic DNA of the selected LAB was extracted and used as template to amplify 16S rDNA gene by the polymerase chain reaction method (PCR) with universal primers as previously described in 3.3.3.2. PCR product size of the partial 16S rDNA gene of NSL13-2, NSL1-4 and NSL5-2 were approximately 1,500 base pairs (bp) as shown in Figure 4.5

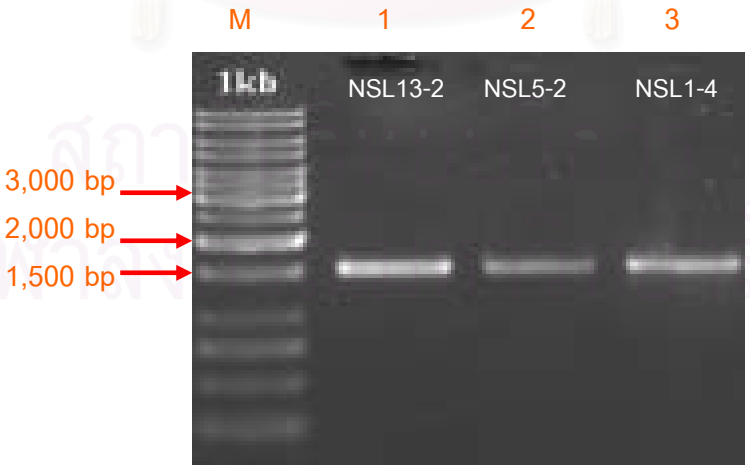


Figure 4.5 Agarose gel electrophoresis of PCR products with universal primers. Genomics DNA were used from: lane 1: NSL13-2; lane 2: NSL5-2; lane 3: NSL1-4. Lane M was loaded with 1 kb ladder DNA markers

Then, sequencing of the partial 16S rDNA gene was commercially serviced by Macrogen Inc. co. Ltd. (Seoul, Korea). The obtained sequences of NSL13-2, NSL5-2 and NSL1-4 were 1,419, 1,463 and 1,446 bp, respectively.

All sequences were blasted and compared alignment with the database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). According to the results, sequencing of the partial 16S rDNA of NSL13-2 (appendix G) gave 99% of identity to the partial 16S rRNA gene of *Pediococcus pentosaceus* strain RTa11, accession number FM179610.1 and E-value 0.0. Five strains from the database that identity to nucleotide sequence of NSL13-2 were shown in Table 4.4.

Table 4.4 Five strains from the database of NCBI that identity to nucleotide sequence of NSL13-2

Accession No.	Strain	%identity
FM179610.1	*Pediococcus pentosaceus* partial 16S rRNA gene, strain RTa11	99
FM179609.1	*Pediococcus pentosaceus* partial 16S rRNA gene, strain RTa4	99
EU569832.1	*Pediococcus pentosaceus* strain KC00716S ribosomal RNA gene, partial sequence	99
EU483113.1	*Pediococcus pentosaceus* strain MY-800 16S ribosomal RNA gene, partial sequence	99
AB362605.1	*Pediococcus pentosaceus* gene for 16S rRNA, partial sequence, strain: NRIC 0123	99

From the results of 16S rDNA analysis, NSL13-2 was identified as *Pediococcus pentosaceus* that corresponded to results of genus identification. The genus *Pediococcus* usually formed in pairs or tetrads (Figure 4.2). A wide range of carbohydrates could be used by this strain such as L-arabinose, ribose, galactose, glucose, fructose, mannose, rhamnose, amygdalin, cellobiose, maltose, melibiose, trehalose and raffinose (Deibel and Niven, 1961) as shown in Table 4.2. Moreover, it was

able to grow at both 10°C and 45°C, but it could not produce CO_2 from glucose fermentation (Table 4.3).

Sequencing of the partial 16S rDNA of NSL1-4 (appendix G) gave 99% of identity to the partial 16S rRNA gene of *Weissella cibaria* strain NRIC 0136, accession number AB362617.1 and E-value 0.0. Five strains from the database that identity to nucleotide sequence of NSL1-4 were shown in Table 4.5.

Table 4.5 Five strains from the database of NCBI that identity to nucleotide sequence of NSL1-4

Accession No.	Strain	%identity
AB362617.1	*Weissella cibaria* gene for 16S rRNA, partial equence, strain: NRIC 0136	99
AB362614.1	*Weissella cibaria* gene for 16S rRNA, partial sequence, strain: NRIC 0133	99
AJ422031.1	*Weissella cibaria* 16S rRNA gene, strain ACA-DC 3411t2	99
DQ294961.1	*Weissella cibaria* strain Uga49-1 16S rRNA gene, complete sequence	99
AM491820.1	*Weissella cibaria* partial 16S rRNA gene, isolate R-32690	99

From the result of 16S rDNA analysis, NSL1-4 was identified as *Weissella cibaria* that corresponded to results of genus identification. *Weissella* was coccobacilli Gram -positive bacteria as shown in Figure 4.3 and a heterofermentative LAB because it could produce CO_2 from glucose fermentation (Table 4.3). Carbohydrates fermentation pattern of this strain showed that it could utilize L-arabinose, esculin, cellobiose, maltose and sucrose. But L-xylose, galactose, melibiose, trehalose and raffinose could not be used by *Weissella* as indicated in Table 4.2 (Collins et al., 1993).

Moreover, sequencing of the partial 16S rDNA of NSL5-2 (appendix G) gave 100%of identity to the partial 16S rRNA gene of *Enterococcus gilvus* strain 2366, accession number EF535229.1 and E-value 0.0. Five strains from the database that identity to nucleotide sequence of NSL5-2 were shown in Table 4.6.

Table 4.6 Five strains from the database of NCBI that identity to nucleotide sequence of NSL5-2

Accession No.	Strain	%identity
EF535229.1	*Enterococcus gilvus* strain 2366 16S ribosomal RNA gene, partial sequence Uncultured bacterium clone	100
AM491815.1	*Enterococcus gilvus* partial 16S rRNA gene, isolate R-31671RNA gene, partial sequence	100
DQ411810.1	*Enterococcus gilvus* strain ATCC BAA-350 16S ribosomal RNA gene	99
EU773759.1	Uncultured bacterium clone CE2_c02_1 16S ribosomal RNA gene, partial sequence	99
EU459323.1	Uncultured bacterium clone HH_aai36d01 16S ribosomal RNA gene, partial sequence	99

From the result of 16S rDNA analysis, NSL5-2 was identified as *Enterococcus gilvus* that corresponded to results of genus identification. The genus *Enterococcus* often occurred in pairs (Figure 4.4). *Enterococcus* was a homofermentative LAB because it could not produce CO_2 from glucose fermentation (Table 4.3). Carbohydrates fermentation pattern of this strain showed that it could utilize ribose, galactose, glucose, fructose, mannose, cellobiose, maltose, lactose, trehalose and gentiobiose as shown in Table 4.2

4.4 Inhibitory efficiency of LAB on pathogenic bacteria in MRS broth

4.4.1 Growth profile and specific growth rate of bacteria in this study

Growth of pathogenic bacteria included *S. aureus* ATCC 25923, *L. monocytogenes* DMST 17303, *B. cereus* ATCC 1729, *S.* Typhimurium ATCC 1331, *E. coli* ATCC 25922 and *P. pentosaceus* NSL13-2 were studied for preparing the inoculum using in the study of the inhibitory efficiency of LAB on pathogenic bacteria in MRS broth.

Growth profiles of *P. pentosaceus* NSL13-2, *S. aureus*, *L. monocytogenes*, *B. cereus*, *E. coli* and *S.* Typhimurium were indicated in Figures F1-F6 (appendix F), their doubling time of 1 hour 13 minutes, 1 hour 4 minutes, 51, 24, 42 and 55 minutes, respectively, were determined. The values of optical density at 600 nm and total viable bacteria count (log $CFUml^{-1}$) were performed in Tables F3-F8 (appendix F).

4.4.2 Investigation of inhibitory efficiency of LAB on pathogenic bacteria by co-culturing in MRS broth

This experiment was divided into three groups including control A, control B and co-culture. Control A and B were MRS broth inoculated with only LAB and pathogenic bacteria, respectively. For co-culture, both of LAB and pathogenic bacteria were inoculated in MRS broth. The initial inoculum of LAB was approximately 10^6 $CFUml^1$ and pathogenic bacteria was 10^4 $CFUml^1$ in all experiments. Sampling was done at 0, 4, 8, 12, 16, 24 and 48 hours in order to determine total viable bacteria counts, pH measurement and bacteriocin detection.

Pediococcus pentosaceus NSL13-2, *Enterococcus gilvus* NSL5-2 and *Weissella cibaria* NSL1-4 were investigated for their inhibitory effect against pathogenic bacteria. For initial study, *S. aureus* and *E. coli* were selected for using as the represent of Gram-positive and Gram-negative bacteria, respectively.

Figure 4.6 (A, B, C) showed the results of co-culture between *P. pentosaceus* NSL13-2 or *E. gilvus* NSL5-2 or *W. cibaria* NSL1-4 and *S. aureus*, respectively.

In Figure 4.6A showed the result of co-culture between *P. pentosaceus* NSL13-2 and *S. aureus* in MRS broth. Growth of *S. aureus* decreased from 4.94 to 4.52 log CFU within 8 hours of incubation and not detected after 12 hours. Moreover, *S. aureus* slightly increased from 4.84 to 5.91 log CFU within 4 hours and was continuously reduced and completely inhibited after 20 hours of incubation when co-cultured with *E. gilvus* NSL5-2 as shown in Figure 4.6B. In addition, *S. aureus* increased from 4.68 to 5.69 log CFU within 4 hours and not detected after 8 hours when co-cultured with *W. cibaria* NSL1-4 as shown in Figure 4.6C.

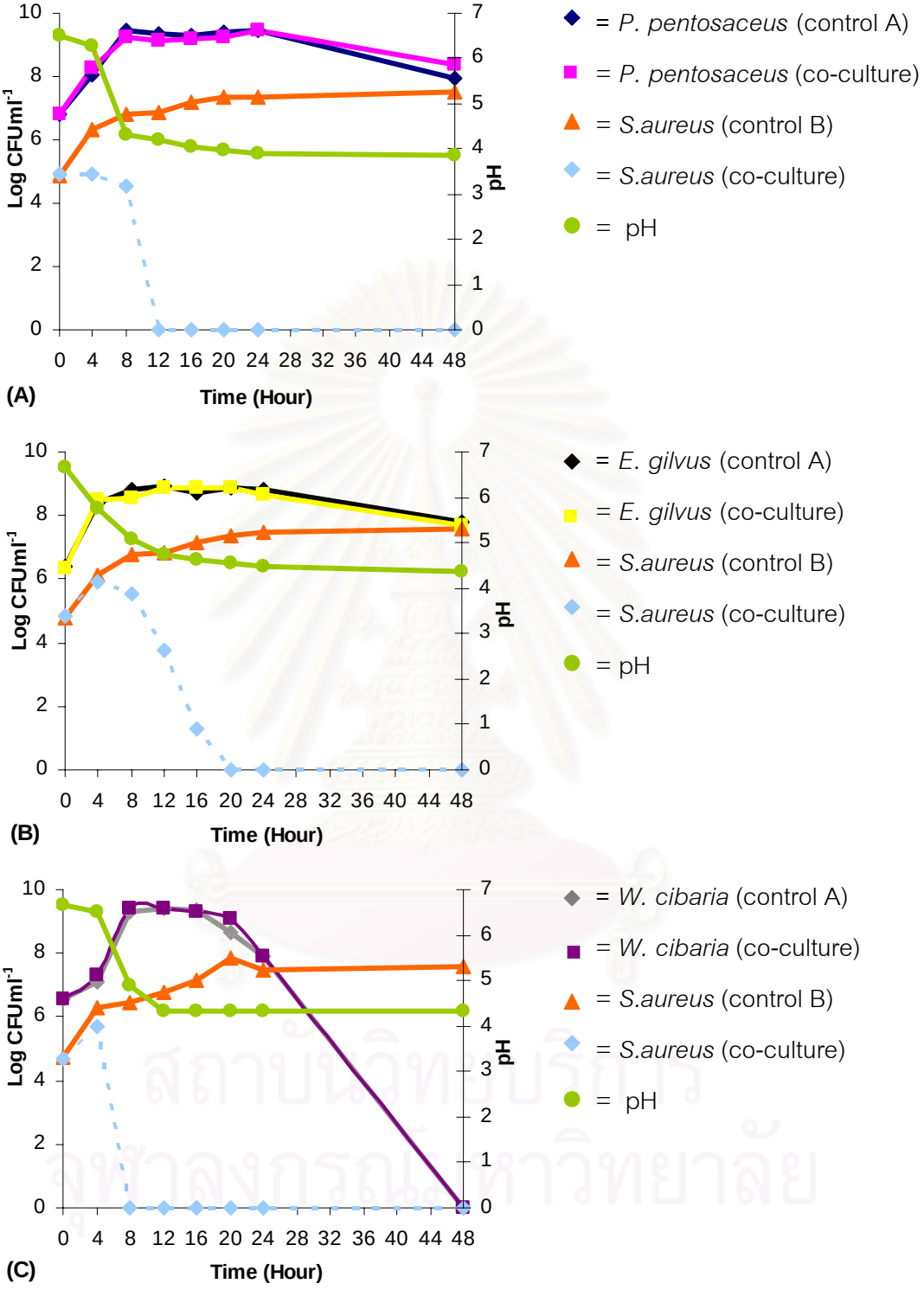


Figure 4.6 Changes in bacteria counts and pH during incubation time when *S. aureus* was co-cultured with *P. pentosaceus* (A); *E. gilvus* NSL5-2 (B); and *W. cibaria* NSL1-4 (C) in MRS broth

Figure 4.7 (A, B, C) showed the results of co-culture between *P. pentosaceus* NSL13-2 or *E. gilvus* NSL5-2 or *W. cibaria* NSL1-4 and *E. coli*, respectively. In Figures 4.7A and B, when *E. coli* was co-cultured with *P. pentosaceus* NSL13-2 or *E. gilvus* NSL5-2, growth of *E. coli* slightly increased to ~5 log CFU at the initial of incubation, after that it was continuously decreased. Complete inhibition of *E. coli* was found after 8 and 16 hours by *W. cibaria* and *P. pentosaceus*, respectively as shown in Figures 4.7C and A. Whereas, *E. gilvus* NSL5-2 showed the same effect at 48 hours (Figure 4.7B).

The number of *P. pentosaceus* NSL13-2 and *W. cibaria* NSL1-4 continuously increased to a maximum of ~9 log CFU within 8 hours and remained constant at ~ 8-9 log CFU until the incubation time was 24 hours, after that it slightly decreased. In the other hand, the maximum number of *E. gilvus* NSL5-2 was ~8 log CFU and remained constant at ~ 8 log CFU until the incubation time was 24 hours, after that it also slightly decreased.

In all experiments, pH of MRS broth continuously decreased when fermentation times increased. pH of co-culture experiments between *P. pentosaceus* NSL13-2 or *E. gilvus* NSL5-2 or *W. cibaria* NSL1-4 and *S. aureus* decreased from 6.52 to 3.86, 6.66 to 4.37 and 6.67 to 4.32, respectively. In addition, pH of co-culture experiments between *P. pentosaceus* NSL13-2 or *E. gilvus* NSL5-2 or *W. cibaria* NSL1-4 and *E. coli* decreased from 6.57 to 3.82, 6.52 to 4.30 and 6.58 to 4.29, respectively. Table F9-F14 (appendix F) showed all total viable bacteria counts and pH of this experiment.

The highest viable bacteria count and lowest pH was observed among the co-cultures of *P. pentosaceus* NSL13-2 with *S. aureus* or *E. coli* (appendix F). From the results, even though *W. cibaria* NSL1-4 could inhibit *S. aureus* and *E. coli* rapidly but it could not survive in MRS broth after 24 hours of incubation. In case of *W. cibaria* NSL1-4, the depletion of nutrients may possibly cause growth inhibition of this strain. Leroy et al. (2001) described that LAB are fastidious with consideration to their nutrient requirements and growth of some strains are often inhibited in MRS medium due to nutrient limitation. Nevertheless, MRS was a commercial medium developed to sustain

growth of LAB and often used for studying. Therefore *P. pentosaceus* was considered as the highest efficiency strain for further study with other pathogenic bacteria.

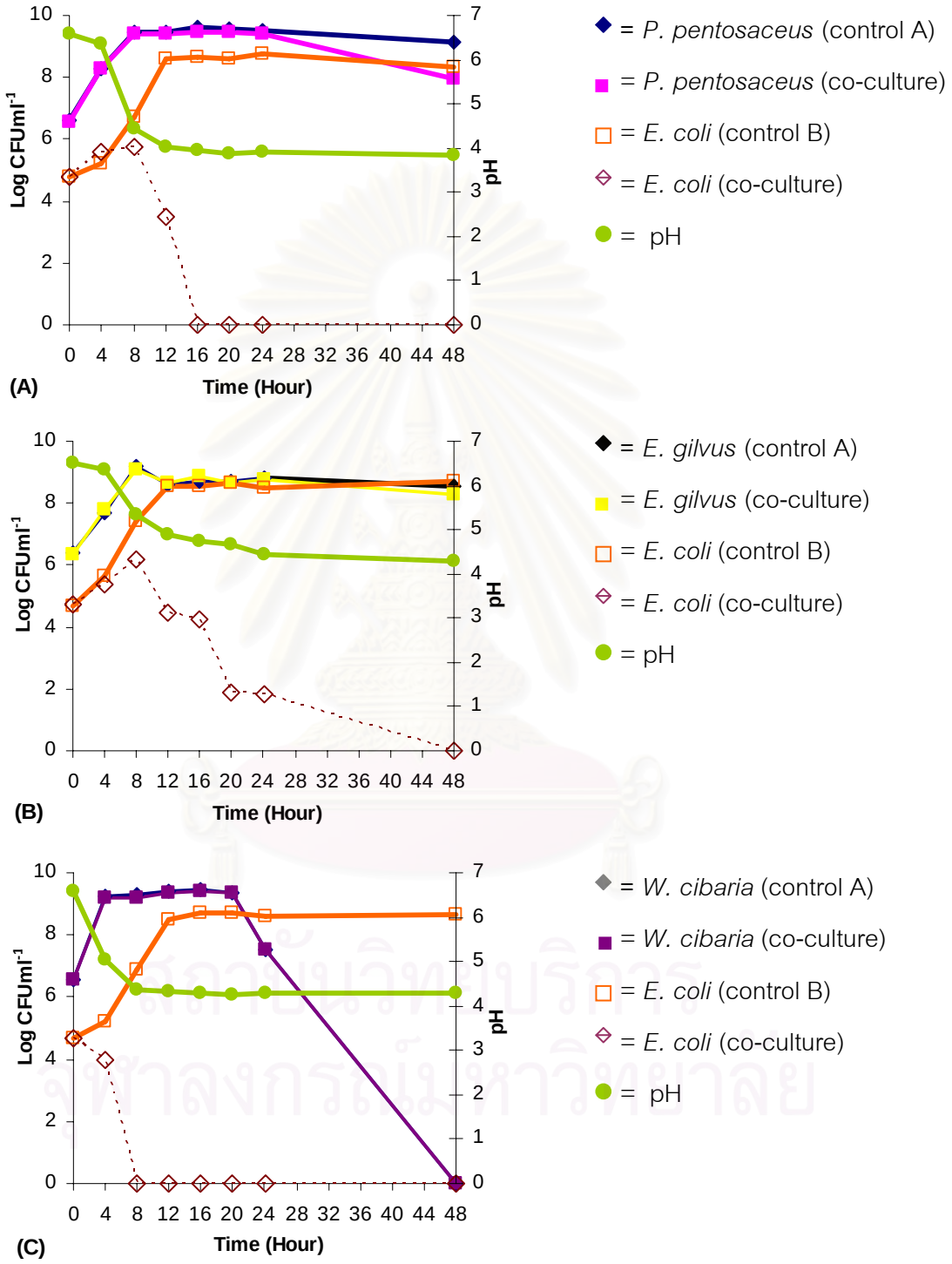


Figure 4.7 Changes in bacteria counts and pH during incubation time when *E. coli* was co-cultured with *P. pentosaceus* (A); *E. gilvus* NSL5-2 (B); and *W. cibaria* NSL1-4 (C) in MRS broth

Cell-free supernatant from all co-culture experimented were determined by using agar well diffusion method with *S. aureus* as an indicator strain. The results showed in Table 4.7, 4.8 and Table 4.9.

Table 4.7 The inhibition zones (cm) of bacteriocin that detected from the co-culture of *P. pentosaceus* NSL13-2 and pathogenic bacteria

Time (hour)	Inhibition zones (cm)	
	P. pentosaceus + *S. aureus* [a]	*P. pentosaceus* + *E. coli* [b]
0	ND	ND
4	1.50 ± 0.00	1.50 ± 0.00
8	1.50 ± 0.00	1.50 ± 0.00
12	1.53 ± 0.06	1.57 ± 0.06
16	1.50 ± 0.00	1.60 ± 0.10
20	1.73 ± 0.23	1.77 ± 0.21
24	1.43 ± 0.12	1.53 ± 0.06
48	1.40 ± 0.17	1.43 ± 0.15

ND = Not detected

a = Inhibition zones were detected from the co-culture of *P. pentosaceus* NSL13-2 and *S. aureus*

b = Inhibition zones were detected from the co-culture of *P. pentosaceus* NSL13-2 and *E. coli*

Table 4.8 The inhibition zones (cm) of bacteriocin that detected from the co-culture of *E. gilvus* NSL5-2 and pathogenic bacteria

Time (hour)	Inhibition zones (cm)	
	E. gilvus NSL5-2 + *S. aureus* [a]	*E. gilvus* NSL5-2 + *E. coli* [b]
0	ND	ND
4	ND	0.00 ± 0.00
8	1.50 ± 0.00	0.00 ± 0.00
12	1.53 ± 0.06	1.13 ± 0.06
16	1.37 ± 0.06	1.20 ± 0.00
20	1.33 ± 0.15	1.27 ± 0.06
24	1.43 ± 0.12	1.30 ± 0.17
48	1.20 ± 0.17	1.57 ± 0.06

ND = Not detected

a = Inhibition zones were detected from the co-culture of *E. gilvus* NSL5-2 and *S. aureus*

b = Inhibition zones were detected from the co-culture of *E. gilvus* NSL5-2 and *E. coli*

Table 4.9 The inhibition zones (cm) of bacteriocin that detected from the co-culture of *W. cibaria* NSL1-4 and pathogenic bacteria

Time (hour)	Inhibition zones (cm)	
	W. cibaria NSL1-4 + *S. aureus* [a]	*W. cibaria* NSL1-4 + *E. coli* [b]
0	ND	ND
4	1.50 ± 0.00	1.47 ± 0.06
8	1.50 ± 0.00	1.50 ± 0.00
12	1.30 ± 0.00	1.27 ± 0.06
16	1.27 ± 0.06	1.27 ± 0.06
20	1.30 ± 0.00	1.33 ± 0.06
24	1.30 ± 0.00	1.20 ± 0.00
48	1.20 ± 0.00	1.23 ± 0.06

ND = Not detected

a = Inhibition zones were detected from the co-culture of *W. cibaria* NSL1-4 and *S. aureus*

b = Inhibition zones were detected from the co-culture of *W. cibaria* NSL1-4 and *E. coli*

Co-cultures of *P. pentosaceus* NSL13-2 with *L. monocytogenes,* or *B. cereus* and *S.* Typhimurium were shown in Figures 4.8 (A, B, C), respectively. At the beginning of incubation, *L. monocytogenes*, *B. cereus* and *S.* Typhimurium slightly increased 1.14, 0.92 and 0.33 log CFU, respectively from initial number. No detection of *L. monocytogenes* and *S.* Typhimurium after 12 hours (Figure 4.8A and C), and *B. cereus* after 8 hours (Figure 4.8B) were found.

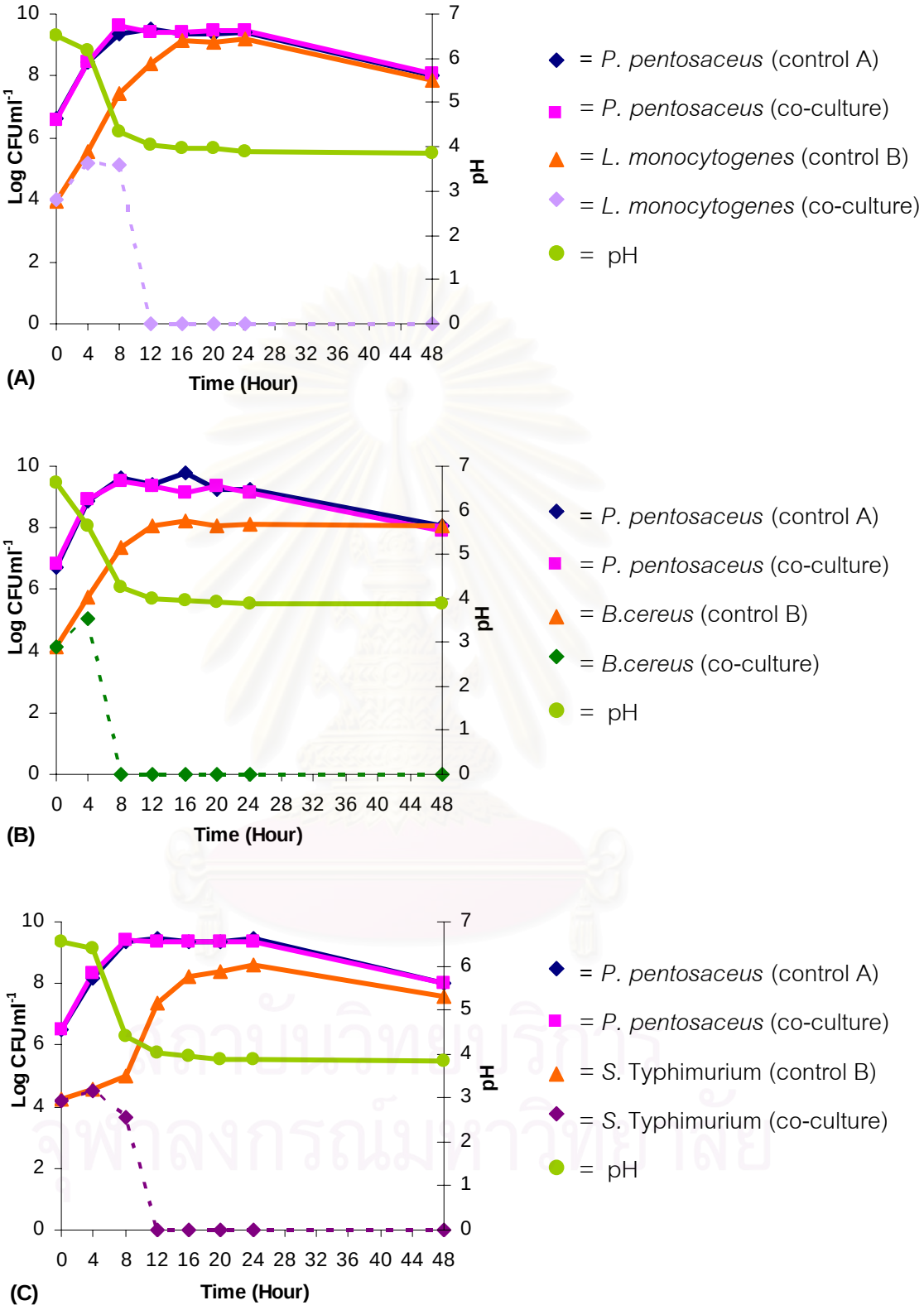


Figure 4.8 Changes in bacteria counts and pH during incubation time when *P. pentosaceus* was co-cultured with (A); *L. monocytogenes* (B); *B .cereus* (C) *S.* Typhimurium in MRS broth

Changes in the number of *P. pentosaceus* NSL13-2 and pH in co-culturing with *L. monocytogenes* were generally similar to the co-culture with *B. cereus* and *S.* Typhimurium as showed in Table F15-F17 in appendix F.

Cell-free supernatant of the co-culture between *P. pentosaceus* NSL13-2 and *L. monocytogenes* or *B. cereus* or *S.* Typhimurium were determined by using agar well diffusion method with *S. aureus* as an indicator strain. The results showed in Table 4.10.

Table 4.10 The inhibition zones (cm) of bacteriocin that detected from the co-culture of *P. pentosaceus* NSL13-2 and pathogenic bacteria

Time (hour)	Inhibition zones (cm)		
	P. pentosaceus NSL13-2 + *L. monocytogenes*[a]	*P. pentosaceus* NSL 13-2 + *B. cereus*[b]	*P. pentosaceus* NSL 13-2 + *S.* Typhimurium[c]
0	ND	ND	ND
4	1.47 ± 0.06	1.33 ± 0.15	1.33 ± 0.06
8	1.50 ± 0.00	1.20 ± 0.00	1.30 ± 0.00
12	1.63 ± 0.06	1.20 ± 0.00	1.43 ± 0.06
16	1.37 ± 0.12	1.23 ± 0.06	1.37 ± 0.06
20	1.40 ± 0.10	1.23 ± 0.06	1.40 ± 0.10
24	1.30 ± 0.00	1.37 ± 0.06	1.53 ± 0.06
48	1.30 ± 0.00	1.30 ± 0.10	1.47 ± 0.06

ND = Not detected

a, b, c = Inhibition zones were detected from the co-culture of *P. pentosaceus* NSL13-2 and *L. monocytogenes, B. cereus* and *S.* Typhimurium, respectively

Co-culture experiments in MRS broth were performed as an *in vitro* for investigating the interaction between selected LAB and certain pathogenic bacteria in order to select the highest efficiency of LAB for using as starter culture in nham preparation. Since raw minced pork is a major composition in nham product that can provide and environment for the growth of pathogenic bacteria. Therefore, starter culture should be produced not only acidic conditions but also some antimicrobial substances like bacteriocin was interested. pH lower than 4.6 could inhibit or protect the growth of some contaminated microorganisms (Vermeiren et al., 2006). Moreover, the remained viable starter culture along fermentation time was required because it was greater competitiveness for nutrients and colonization that gave slower growth or inhibited some competitors (Bredholt et al., 1999).

Compare the acid production ability of 3 strains, pH lower than 4.6 was achieved within 8 hours of incubation time in co-culturing between *P. pentosaceus* NSL13-2 and all pathogenic bacteria. The co-culture of *E. gilvus* NSL5-2 with *S. aureus* or *E. coli* gave a desired pH at 16 and 24 hours, respectively. Moreover, a desired pH was achieved at 8 and 12 hours in co-culturing between *W. cibaria* and *E. coli* or *S. aureus*. At the end of incubation, the lowest pH was observed among the co-cultures of *P. pentosaceus.*

The numbers of *P. pentosaceus* NSL13-2 in all co-culture groups were remained approximately 8 log CFU at the end of incubation that were higher than *E. gilvus* NSL5-2 and *W. cibaria* in the same experiment. In addition, bacteriocin production were found in all experiments.

Moreover, pattern of carbohydrates fermentation of selected LAB was examined because heterofermentative LAB are not suitable for nham production because the formation of large amounts of carbon dioxide gas leads to holes of different sizes in the product and these LAB produce concentrations of acetic acid that cause a pungent off-flavor (Buckenhüskes, 1993). From Table 4.3 could imply that *P. pentosaceus* NSL13-2 and *E. gilvus* NSL5-2 are a homofermentative LAB. In contrast, *W. cibaria* is a heterofermentative LAB because it can produce carbon dioxide

gas from glucose fermentation. So, it was not suitable for using as starter culture in nham preparation.

Although Enterococci may be applied as starter cultures in some foods and are commercially available as probiotic cultures for protecting and treating intestinal disorders in animals and humans (Stiles and Holzapfel, 1997). However, this genus may also be negatively associated with foods due to it is an indicator of faecal (Franz et al., 1999). Therefore, *P. pentosaceus* NSL13-2 had appropriate properties for applying as starter culture in this research.

4.5 Evaluation of environmental conditions for production of bacteriocin

Bacteriocin production could be influenced by culture conditions such as temperature, pH, NaCl concentration and incubation time (Gänzle et al., 1999). In order to find appropriate conditions for bacteriocin production, the effect of various conditions on bacteriocin activity of *P. pentosaceus* NSL13-2 was observed by using MRS broth as a culture medium.

4.5.1 Effect of temperature on the bacteriocin production

Different temperature including 25°C, 30°C, 35°C, 37°C, 40°C, and 45°C were studied. The result showed in Table 4.11.

Table 4.11 Effect of temperature on the bacteriocin activity of *P. pentosaceus* NSL13-2

Temperature	Growth (OD_{600})	Final pH	Bacteriocin activity ($AUml^{-1}$)
25	1.943 ± 0.027	3.99 ± 0.01	400
30	1.948 ± 0.007	3.92 ± 0.02	400
35	1.921 ± 0.023	3.91 ± 0.01	400
37	1.904 ± 0.015	3.90 ± 0.01	400
40	1.818 ± 0.038	4.06 ± 0.01	200
45	1.184 ± 0.038	4.21 ± 0.02	200

Growth of *P. pentosaceus* NSL13-2 was observed from the optical density at 600 nm (OD_{600}). From Table 4.11 showed that it was able to grow at all temperature ranges, but the reduction of growth was observed when culture was incubated at temperature up to 40-45°C. The optimal growth temperature was 30°C. At all temperature, reduction of pH was given the same trend and the lowest pH obtained by incubation at 37°C. High temperature was the factor that reduced bacteriocin production or activity. Ranges of temperature from 25 to 37°C were more favourable for growth and bacteriocin production. This result corresponded to the research of Settanni et al. (2008), who studied the role of environmental factors on bacteriocin-like inhibitory substances (BLIS) production by *Enterococcus mundtii* strains. Their results concluded that the temperature for maximum BLIS were represented by MRS as growth medium and incubation between 30 and 37°C, therefore conditions for high BLIS productions may possible coincide with those for optimal growth.

Although growth obtained from the incubation at 37°C was slightly lower than at 30°C but this condition gave both of high acid production and the same of bacteriocin activity, so 37°C was chosen as temperature for further experiments.

4.5.2 Effect of pH on the bacteriocin production

P. pentosaceus NSL13-2 grew well in pH range 7.0-9.5. But the optimal growth was at pH 8.0. However, the high bacteriocin activity was observed at pH 6.0, 7.0 and 9.5 in culture broth as shown in Table 4.12. Although, maximum growth exhibited at pH 8.0, bacteriocin was not detected of which the same result observed at pH 7.5. Therefore, bacteriocin activities were not always correlated with cell concentration or growth rate of producer strain as mentioned earlier (Kim et al., 1997). Also, higher levels of bacteriocin production were given at conditions that differed from those required for optimal growth (Parente and Ricciardi, 1994).

Moreover, pH was associated with the absorption of bacteriocin molecules to cell wall. Higher degree of absorption was increased when pH increased (Leroy and De Vuyst, 1999). From the result, higher bacteriocin activity was also observed at pH 9.5

because it showed the highest ▲pH which referred to acid production during incubation time that resulted in increasing of released bacteriocin molecules.

Table 4.12 Effect of pH on the bacteriocin activity of *P. pentosaceus* NSL13-2

Initial pH	Growth (OD_{600})	Final pH	▲pH	Bacteriocin activity ($AUml^{-1}$)
4.00	0.899 ± 0.032	3.68 ± 0.05	0.32 ± 0.05	100
5.00	1.504 ± 0.019	3.86 ± 0.07	1.14 ± 0.07	200
6.00	1.610 ± 0.003	3.90 ± 0.03	2.10 ± 0.03	400
7.00	1.904 ± 0.022	3.93 ± 0.01	3.07 ± 0.01	400
7.50	1.973 ± 0.003	3.93 ± 0.04	3.57 ± 0.04	-
8.00	2.005 ± 0.002	3.98 ± 0.03	4.02 ± 0.03	-
9.00	1.999 ± 0.077	4.02 ± 0.03	4.98 ± 0.03	200
9.50	1.956 ± 0.027	4.14 ± 0.06	5.36 ± 0.06	400

4.5.3 Effect of NaCl on the bacteriocin production

Monitoring various NaCl concentration including 0, 1, 3, 5, 7, 9, 11, 13 and 15 % (w/v) in culture broth on growth and bacteriocin production were performed. The result showed in Table 4.13. *P. pentosaceus* NSL13-2 grew well in NaCl concentration range 0-3%. Growth continuously decreased when NaCl concentration increased. Optimal growth was observed when culture was grown in MRS broth without NaCl. But the highest bacteriocin activity was obtained by adding NaCl at 3%. Whereas, NaCl concentration higher than 3%, reduction of bacteriocin activity was found which corresponded to the search of Settanni et al. (2008), that was no growth and bacteriocin occurred for *Enterococcus mundtii* WGWT1-1A when NaCl concentration increased up

to 8%. This evidence was also found by Leroy et al. (2003) that salt stress decreased cell growth and specific bacteriocin production of *E. faecium* RZS C5.

Table 4.13 Effect of NaCl on the bacteriocin activity of *P. pentosaceus* NSL13-2

NaCl (%w/v)	Growth (OD_{600})	Final pH	Bacteriocin activity ($AUml^{-1}$)
0	1.928 ± 0.015	3.90 ± 0.01	400
1	1.904 ± 0.006	3.92 ± 0.02	400
3	1.854 ± 0.049	3.95 ± 0.02	800
5	1.148 ± 0.081	4.11 ± 0.01	100
7	0.835 ± 0.050	4.22 ± 0.01	0
9	0.205 ± 0.007	5.00 ± 0.02	0
11	0.030 ± 0.001	5.72 ± 0.01	0
13	0.010 ± 0.002	5.81 ± 0.01	0
15	0.002 ± 0.001	6.13 ± 0.02	0

4.5.4 Effect of incubation time on the bacteriocin production

Different incubation times including 12, 24 and 36 hours were studied. The result showed in Table 4.14. Incubation time at 12, 24 and 36 were represented the growth in late logarithmic, stationary and decline phase of *P. pentosaceus* NSL 13-2 when compared with growth curve graph in Figure F6 (appendix F). Maximal bacteriocin activity was found at 12 and 24 hours of incubation time. From this result could describe that bacteriocin was a secondary metabolites because it was produced in late logarithmic and stationary phase that not involved the normal growth of cell. However, some reports indicated that bacteriocin was produced throughout the experimental growth phase and not solely during logarithmic or early stationary phase (Joerger and Klaenhammer, 1986; Piard et al., 1990)

After stationary phase (at 36 hours), growth and bacteriocin activity were decreased. Decrease of bacteriocin activity might be due to the activity of extracellular endogenous proteinase induced during this phase (Ogunbanwo et al. 2003).

Table 4.14 Effect of incubation time on the bacteriocin activity of *P. pentosaceus* NSL13-2

Incubation time (hour)	Growth (OD_{600})	Final pH	Bacteriocin activity ($AUml^{-1}$)
12	1.577 ± 0.004	4.05 ± 0.01	400
24	1.901 ± 0.003	3.86 ± 0.01	400
36	1.735 ± 0.001	3.84 ± 0.02	200

Optimal of microbial growth and bacteriocin production were also be dependent on the influence of several factors such as temperature, pH, NaCl and incubation time that also involved with nham production. This knowledge could be used as the data for consideration of selected LAB that with or without proper properties to apply as starter culture in nham production. In this experiment, *P. pentosaceus* NSL13-2 gave the highest growth at 30°C which was temperature for nham fermentation. Moreover, it was able to grow in various conditions of pH and NaCl concentration, thus this strain could adapt itself for survival in finished product. Therefore, *P. pentosaceus* NSL13-2 should applicably be used as starter culture in nham preparation.

Except all of conditions that described above, bacteriocin production was strongly dependent on the composition and concentration of complex nutrients. In general, MRS medium was usually used to study of LAB, because it had an essential nutrient to support the growth of LAB. However, nutrient limitation was the problem of MRS that caused growth inhibition in some strains of LAB (Leroy et al., 2001). In addition, bacteriocin was produced when nutrients were available for metabolic activity.

The influence of nutrients on bacteriocin production was immensely reported. Nel et al. (2001) described that pediocin PD-1 from *Pediococcus damnosus*

NCFB 1832 was not only stimulated by the lowering in pH during growth, but might also be stimulated by the presence of specific growth factors (1.7% w/v bacteriological peptone, 0.014%w/v $MnSO_4$ or 3%v/v Tween 80). Ogunbanwo et al. (2003) reported that higher amounts of bacteriocin were synthesized when MRS medium was supplemented with 1.0% glucose, 0.5% Tween 80, 2-3.0%yeast extract and 1-2.0%NaCl, while the supplementation of tri-ammonium, citrate, sodium acetate, magnesium sulphate, manganese sulphate and potassium phosphate had no effect on bacteriocin production. So, modification of nutrients of cultivation media was the alternative way for maximal production of bacteriocin.

4.6 Inhibitory efficiency of LAB on pathogenic bacteria in nham as food model

P. pentosaceus NSL13-2 selected from the experiment of inhibitory efficiency on pathogenic bacteria in MRS broth was used as starter culture for study inhibitory efficiency in nham as food model in this study.

The experimental of nham were divided into 4 batchs including NNF (naturally fermented nham as a control), NS (nham inoculated with 10^6 $CFUg^{-1}$ of LAB), NP (nham inoculated with 10^4 $CFUg^{-1}$ of pathogenic bacteria) and NSP (nham inoculated with 10^6 $CFUg^{-1}$ of LAB and 10^4 $CFUg^{-1}$ of pathogenic bacteria). Nham samples were randomly taken at 0, 24, 72, 120 and 168 hours of incubation time.

4.6.1 Inhibitory efficiency of LAB on *S. aureus* in nham

Profile of LAB population in nham inoculated with 10^6 $CFUg^{-1}$ of *P. pentosaceus* (NS) was similar to naturally fermented nham (NNF) (Figures 4.9A and B), and those from previous research of Visessanguan et al. (2006). Initial LAB flora of nham derived mainly from the raw materials. However, the initial level of LAB in NS was slightly higher than NNF. Number of LAB counts in NS and NNF were 6.82 and 5.83 log CFU, respectively at 0 hour. From the results, the number of LAB in NNF and NS increased to a maximum of ~9 log CFU within 24 hours and remained constant until 120

hours of fermentation time, after that reduction in LAB counts was observed with increasing fermentation time to 168 hours. Changes of LAB and total viable bacteria counts (TVC) of NNF and NP showed the same trend. TVC of NNF and NP were found to increase from 6.25 to 9.27 log CFU and 6.17 to 9.21 log CFU, respectively at 24 hours. The reduction in TVC of NNF and NP decreased to 7.79 and 7.73 log CFU, respectively at 168 hours of fermentation time. Moreover, LAB and TVC in nham inoculated with 10^6 $CFUg^{-1}$ of *P. pentosaceus* and 10^4 $CFUg^{-1}$ of *S. aureus* (NSP) expressed the same trend with NS. TVC of NS and NSP were found to increase from 6.43 to 8.44 log CFU and 6.11 to 8.54 log CFU, respectively at 24 hours and the reduction in TVC of NS and NSP decreased to 7.52 and 7.34 log CFU, respectively at 168 hours of fermentation time.

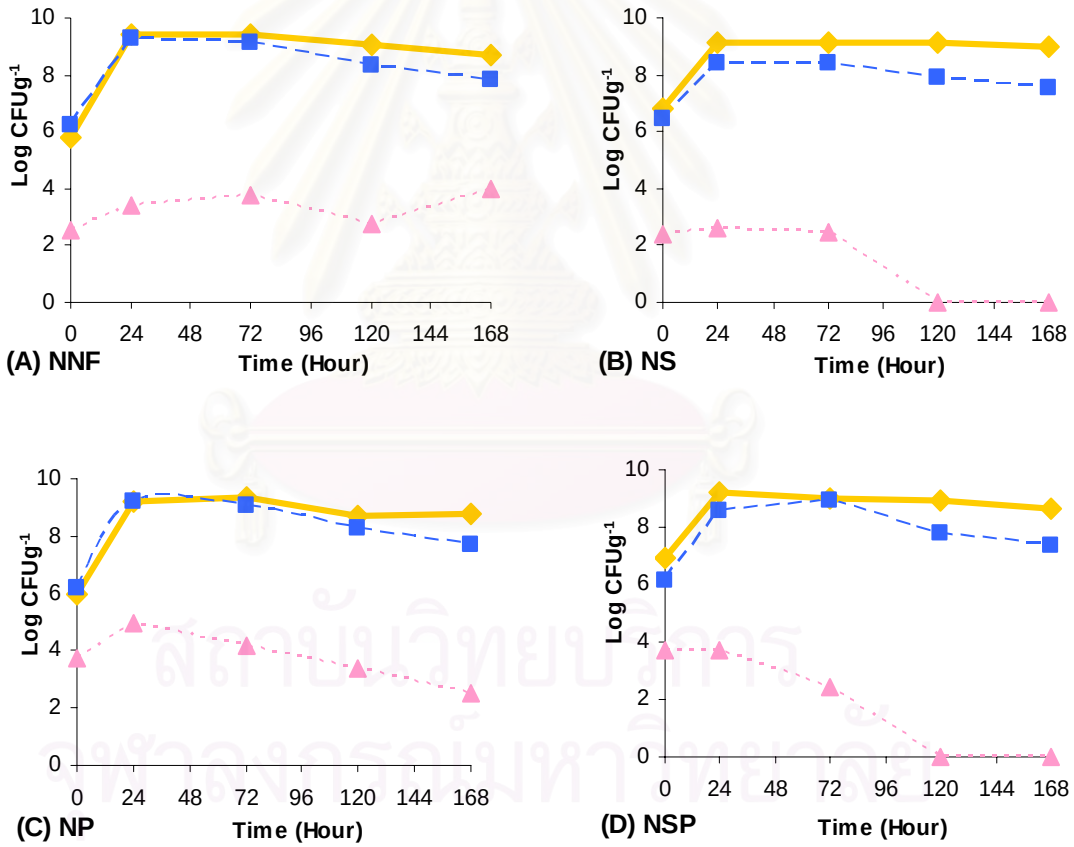


Figure 4.9 Changes in bacteria counts in nham during fermentation. (A) NNF: naturally fermented nham as a control; (B) NS: nham inoculated with *P. pentosaceus*; (C) NP: nham inoculated with *S. aureus* and (D) NSP: nham inoculated with *P. pentosaceus* and *S. aureus*. LAB (◆); Total viable bacteria counts (■); *S. aureus* (▲)

S. aureus in NP and NSP increased from 3.71 to 4.96 log CFU and 3.68 to 3.70 log CFU, respectively within 24 hours of fermentation time as shown in Figures 4.9C and D. No detection of *S. aureus* was observed in NS and NSP at 120 hours. Obviously, *S. aureus* were also detected in NP and NNF over the time of fermentation (Figures 4.9A and C) which were assumed their presence as flora from raw materials. Since *S. aureus* often presented in fresh meat that related to human handling, meat handling surfaces and careless of slaughter (Robert, 1982).

Changes in pH of nham including NNF, NS, NP and NSP were shown in Figure 4.10A. Total acid was shown in term of relative of total acid increase (Figure 4.10B). Because *P. pentosaceus* which used as starter culture in this experiment was homofermentative-LAB, so calculation of %total acid of NS and NSP in all experiments was done based on lactic acid production. During fermentation, pH continuously decreased to the acidic range, while relative of total acid was increased. The pH decreased from 5.7 to 4.8 within 24 hours and reached to pH 4.4 within 72 hours for all nham models. At the end of fermentation, NS and NSP exhibited a lower pH than NNF and NP. The final pH of NS, NSP, NNF and NP were 4.30, 4.28, 4.35 and 4.33, respectively. Changes in bacteria counts, pH and relative of total acid increase (%w/v) were shown in Table F18 (appendix F).

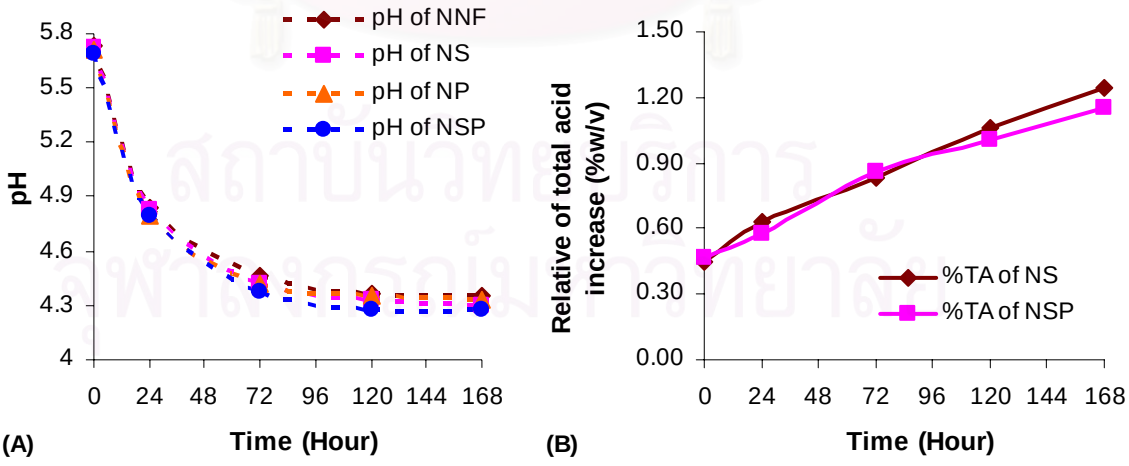


Figure 4.10 Changes in pH (A) and relative of total acid increase (%w/v) based on lactic acid production (B) of nham as a food model in the study of inhibitory effect on *S. aureus* during fermentation

4.6.2 Inhibitory efficiency of LAB on *L. monocytogenes* in nham

Changes of LAB and total viable bacteria counts (TVC) in naturally fermented nham (NNF) were generally similar to nham inoculated with *L. monocytogenes* (NP), while nham inoculated with *P. pentosaceus* (NS) performed the same trend with nham inoculated with both of *P. pentosaceus* and *L. monocytogenes* (NSP). The number of LAB counts in NNF, NS, NP and NSP were 5.15, 6.76, 5.31 and 6.64 log CFU, respectively at 0 hour. From the results, the number of LAB in all batches increased to a maximum of ~9 log CFU within 24 hours, after that LAB counts slightly decreased with increasing fermentation time to 168 hours (Figure 4.11).

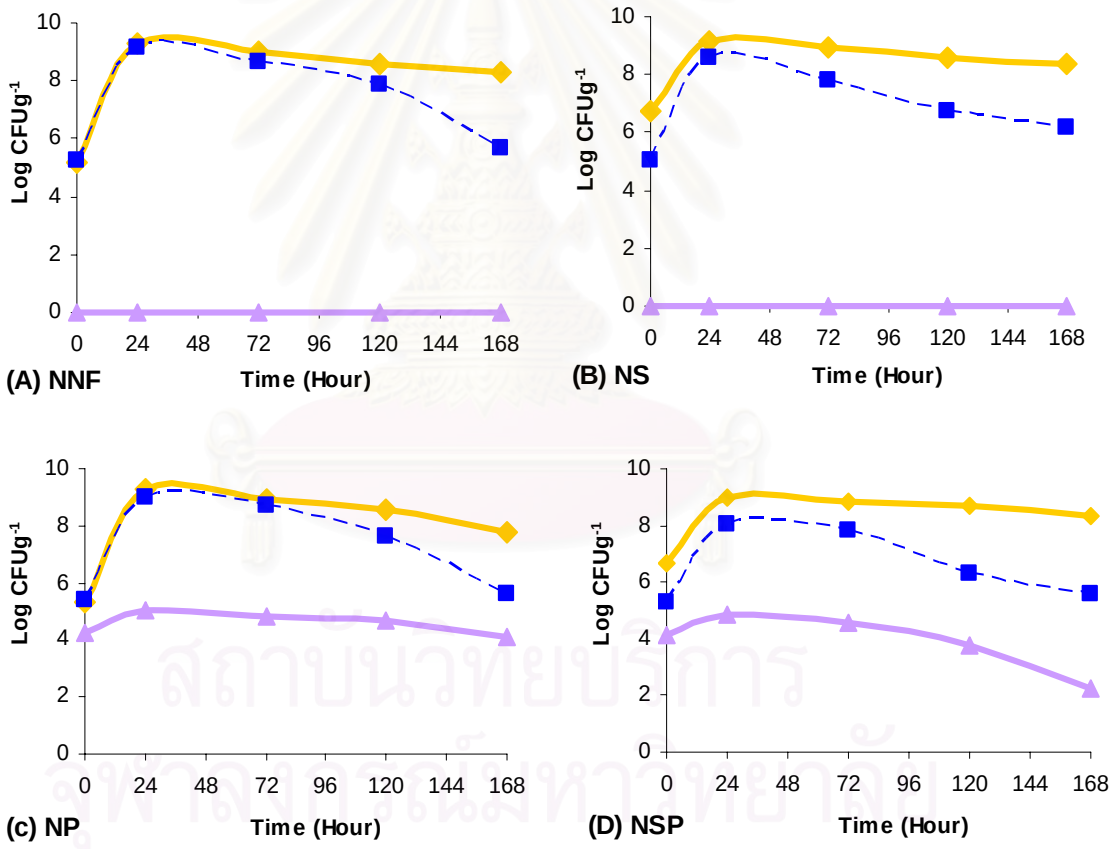


Figure 4.11 Changes in bacteria counts in nham during fermentation. (A) NNF: naturally fermented nham as a control; (B) NS: nham inoculated with *P. pentosaceus*; (C) NP: nham inoculated with *L. monocytogenes* and (D) NSP: nham inoculated with *P. pentosaceus* and *L. monocytogenes*. LAB (◆); Total viable bacteria counts (■); *L. monocytogenes* (▲)

TVC of NNF and NP were found to increase 3.84 and 3.64 log CFU from the initial number, respectively at 24 hours, while NS and NSP increased 2.52 and 2.75, respectively. The reduction in TVC of NNF, NS, NP and NSP decreased to 5.67, 6.19, 5.62 and 5.57 log CFU, respectively at 168 hours of fermentation time.

Due to no detection of *L. monocytogenes* in NNF and NS, presence of *L. monocytogenes* in NP and NSP were only obtained from the inoculation. From Figures 4.11C and D showed the same trend of *L. monocytogenes* growth in NP and NSP. Its growth slightly increased from 4.25 to 5.03 log CFU in NP and from 4.10 to 4.87 log CFU in NSP. However, NSP showed the lower of *L. monocytogenes* count than NP at complete fermentation time. In NSP, *L. monocytogenes* decreased to 2.26 log CFU, while it remained constant at ~4 log CFU in NP.

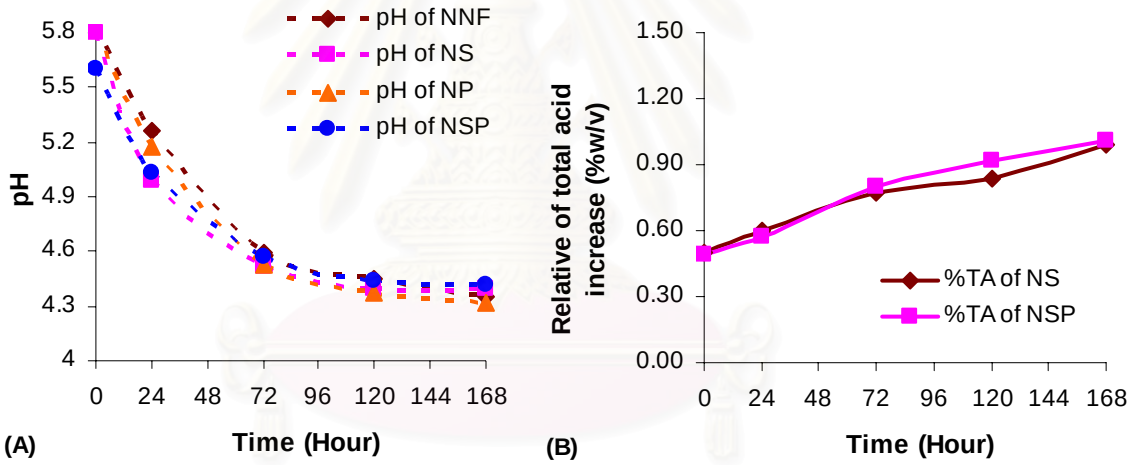


Figure 4.12 Changes in pH (A) and relative of total acid increase (%w/v) based on lactic acid production (B) of nham as a food model in the study of inhibitory effect on *L. monocytogenes* during fermentation

Changes in pH and relative of total acid increase (%w/v) of nham samples were shown in Figure 4.12A and B, respectively. During fermentation, pH continuously decreased to the acidic range, while relative of total acid was increased. Final pH of NNF, NS, NP and NSP were 4.35, 4.39, 4.32 and 4.42, respectively. Final relative of total acid increase of NS and NSP based on lactic acid production were 0.99 and 1.01,

respectively. Changes in bacteria counts, pH and relative of total acid increase (%w/v) were shown in Table F19 (appendix F).

4.6.3 Inhibitory efficiency of LAB on *B. cereus* in nham

All nham samples showed the same trend in change of LAB counts. The initial number of LAB in NNF, NS, NP and NSP were 4.68, 6.94, 4.87 and 6.97 log CFU, respectively. From Figure 4.13, the number of LAB in all batches increased to a maximum of ~9 log CFU within 24 hours and remained constant until 72 hours of fermentation time, after that LAB counts slightly decreased with increasing fermentation time to 168 hours.

Change in TVC of NNF and NP showed the same trend, while NS and NSP also showed the same trend. TVC of NNF and NP were found to increase 4.06 and 3.98 log CFU from the initial number, respectively at 24 hours, while NS and NSP increased to 2.97 and 2.86 log CFU, respectively. At the end of fermentation, NS and NSP exhibited a lower TVC than NNF and NP. Final TVC of NNF, NS, NP and NSP were 6.97, 6.64, 7.27 and 6.60, respectively.

Because of no detection of *B. cereus* in NNF and NS, presence of *B. cereus* in NP and NSP were only obtained from the inoculation. No detection of *B. cereus* was observed in both of NP and NSP after 24 hours of incubation time as shown in Figures 4.13 C and D.

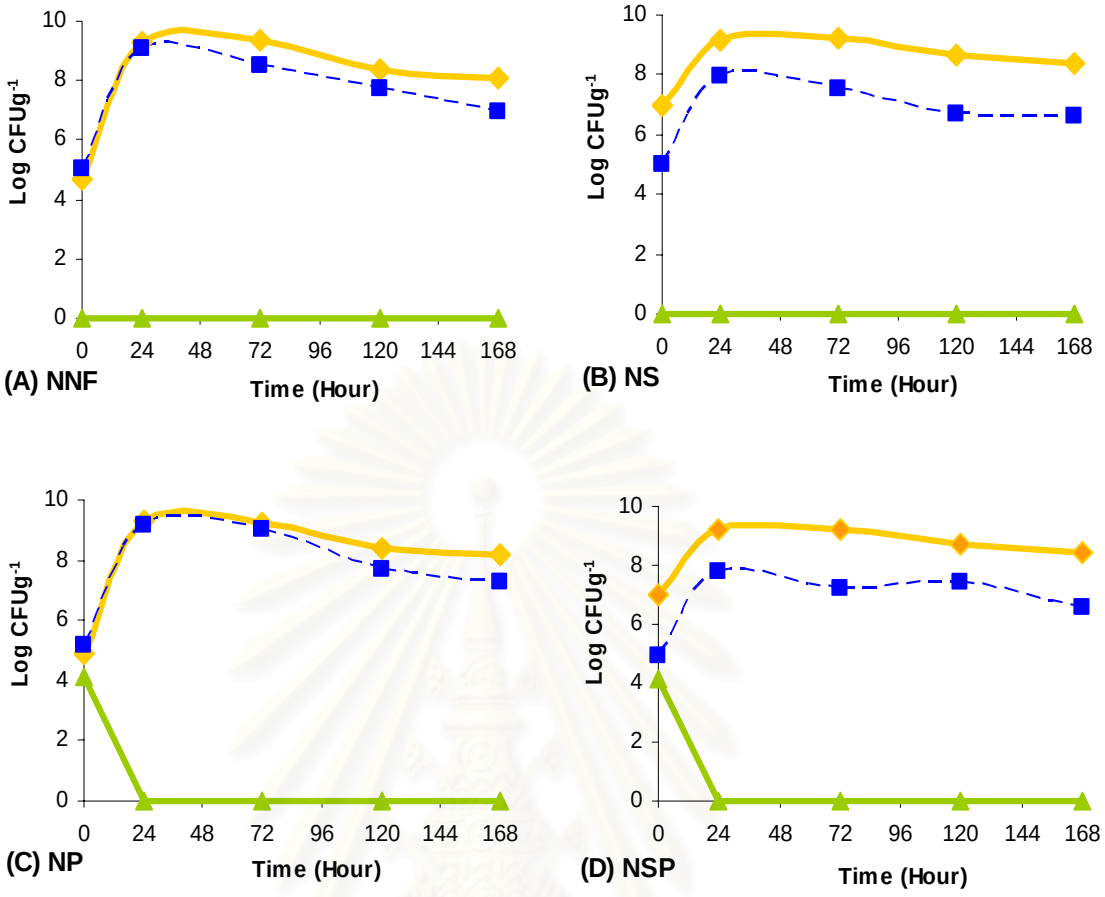


Figure 4.13 Changes in bacteria counts in nham during fermentation. (A) NNF: naturally fermented nham as a control; (B) NS: nham inoculated with *P. pentosaceus*; (C) NP: nham inoculated with *B. cereus* and (D) NSP: nham inoculated with *P. pentosaceus* and *B. cereus*. LAB (◆); Total viable bacteria counts (■); *B. cereus* (▲)

Changes in pH and relative of total acid increase (%w/v) of nham samples were shown in Figure 4.14A and B, respectively. During fermentation, pH continuously decreased that corresponded with the increase of relative of total acid. Final pH of NNF, NS, NP and NSP were 4.39, 4.41, 4.28 and 4.40, respectively. Final relative of total acid increase of NS and NSP based on lactic acid production were 1.14 and 1.01, respectively. Changes in bacteria counts, pH and relative of total acid increase (%w/v) were shown in Table F20 (appendix F).

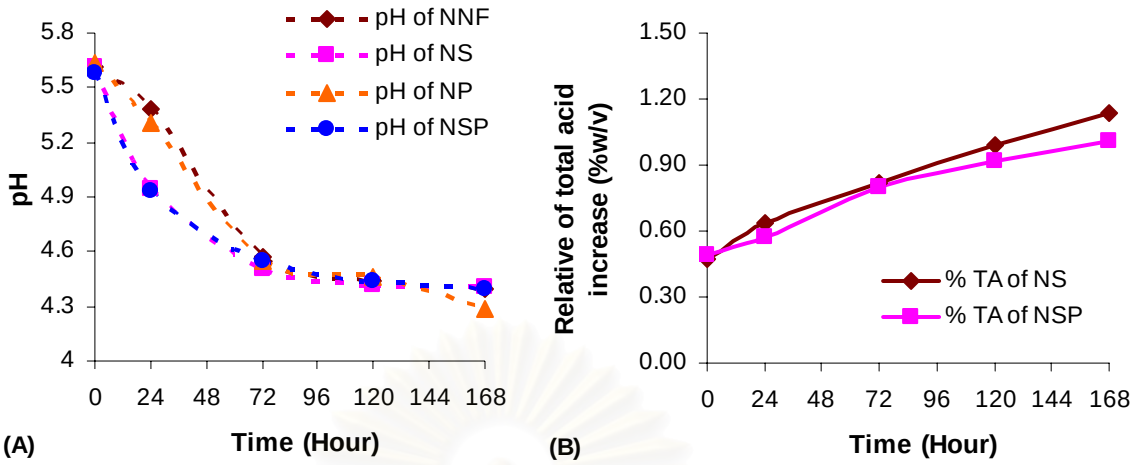


Figure 4.14 Changes in pH (A) and relative of total acid increase (%w/v) based on lactic acid production (B) of nham as a food model in the study of inhibitory effect on *B. cereus* during fermentation

4.6.4 Inhibitory efficiency of LAB on *E. coli* in nham

In all nham samples showed the same trend in change of LAB counts. The initial number of LAB in NNF, NS, NP and NSP were 5.39, 6.83, 5.74 and 6.82 log CFU, respectively. The number of LAB in all batches increased to a maximum of ~9 log CFU within 24 hours and remained constant until 72 hours of fermentation time, after that LAB counts slightly decreased with increasing fermentation time to 168 hours (Figure 4.15).

Increase in the number of TVC in NNF and NP were higher than NS and NSP at 24 hour of fermentation time. TVC of NNF and NP increased to 9.47 and 9.41 log CFU, respectively, while NS and NSP increased to 8.39.and 8.20, respectively. At the end of fermentation, NS and NSP exhibited a lower TVC than those of NNF and NP. The final TVC of NNF, NS, NP and NSP were 7.15, 6.47, 7.24 and 6.92, respectively.

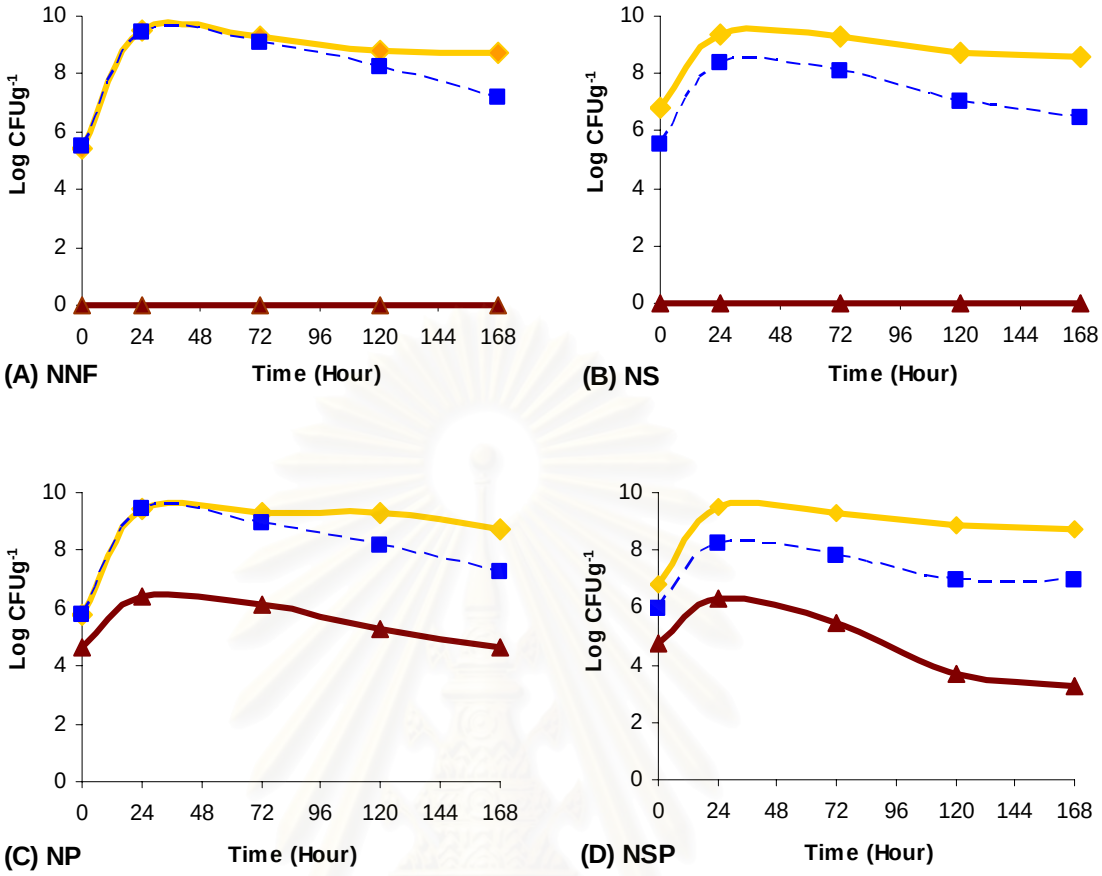


Figure 4.15 Changes in bacteria counts in nham during fermentation. (A) NNF: naturally fermented nham as a control; (B) NS: nham inoculated with *P. pentosaceus*; (C) NP: nham inoculated with *E. coli* and (D) NSP: nham inoculated with *P. pentosaceus* and *E. coli*. LAB (◆); Total viable bacteria counts (■); *E.coli* (▲)

No detection of *E. coli* in NNF and NS. Therefore, the presence of *E. coli* in NP and NSP were only obtained from the inoculation. From Figures 4.15C and D showed the same trend of *E. coli* growth in NP and NSP. At the beginning of fermentation time, growth of *E. coli* slightly increased from 4.65 to 6.44 log CFU in NP and from 4.75 to 6.31 log CFU in NSP. However, NSP showed the lower of *E. coli* count than NP at complete fermentation time. In NSP, *E. coli* decreased to 3.23 log CFU, while it remained constant at ~4 log CFU in NP.

Changes in pH and relative of total acid increase (%w/v) of nham samples were shown in Figure 4.16A and B, respectively. During fermentation, pH continuously decreased that corresponded with the increase of %relative total acidity. Final pH of NNF, NS, NP and NSP were 4.56, 4.39, 4.54 and 4.42, respectively. Final relative of total acid increase of NS and NSP based on lactic acid production were 0.99 and 1.01, respectively. Changes in bacteria counts, pH and relative of total acid increase (%w/v) were shown in Table F21 (appendix F).

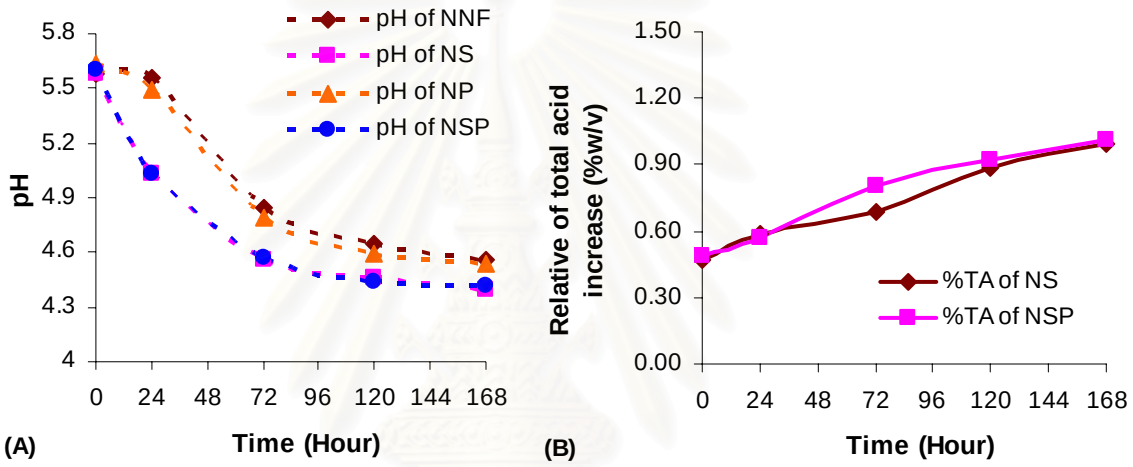


Figure 4.16 Changes in pH (A) and relative of total acid increase (%w/v) based on lactic acid production (B) of nham as a food model in the study of inhibitory effect on *E. coli* during fermentation

4.6.5 Inhibitory efficiency of LAB on *S.* Typhimurium in nham

Same profile of LAB growth was found in all nham samples. The initial number of LAB in NNF, NS, NP and NSP were 5.03, 6.88, 5.04 and 6.75 log CFU, respectively. The number of LAB in all batches increased to a maximum of ~9 log CFU within 24 hours, after that LAB counts slightly decreased and remained constant at~8 log CFU until the end of fermentation time (Figure 4.17).

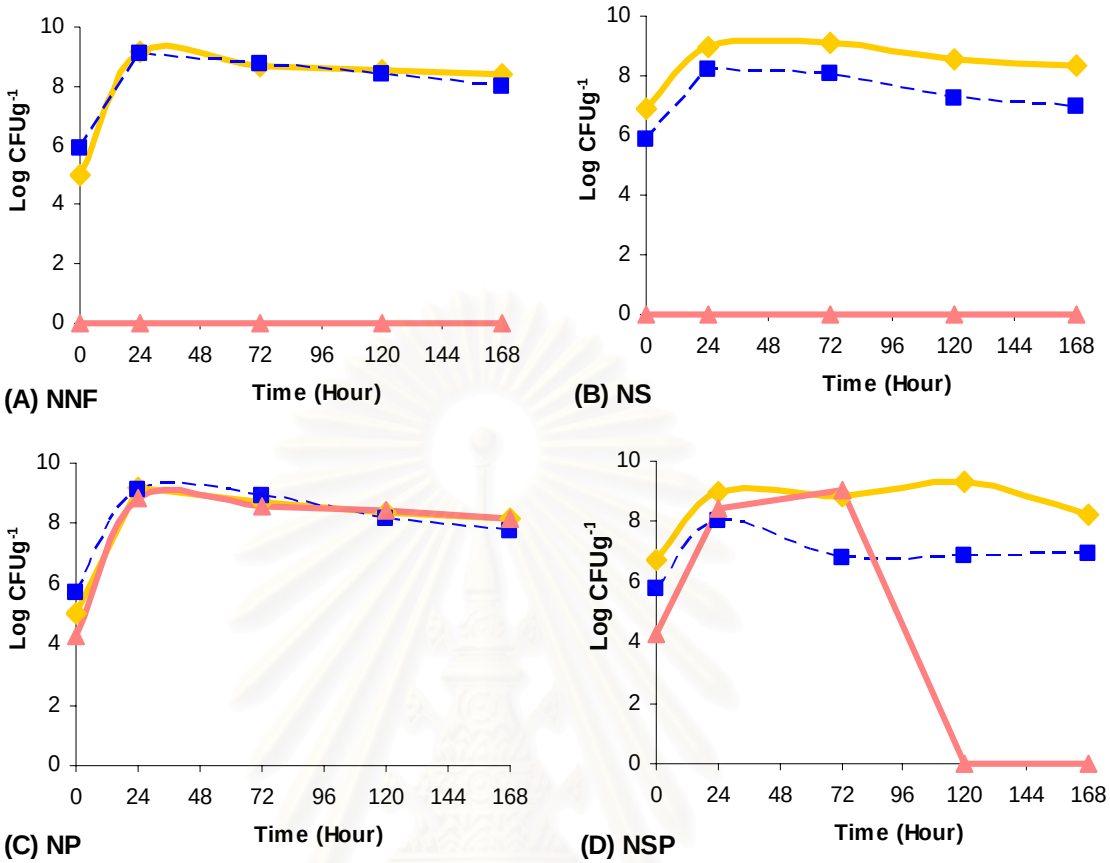


Figure 4.17 Changes in bacteria counts in nham during fermentation. (A) NNF: naturally fermented nham as a control; (B) NS: nham inoculated with *P. pentosaceus*; (C) NP: nham inoculated with *S.* Typhimurium and (D) NSP: nham inoculated with *P. pentosaceus* and *S.* Typhimurium. LAB (◆); Total viable bacteria counts (■); *S.* Typhimurium (▲)

Increase in the number of TVC in NNF and NP were higher than those of NS and NSP at 24 hours of fermentation time. TVC of NNF and NP increased to 9.12 and 9.13 log CFU, respectively, while NS and NSP increased to 8.19.and 8.05, respectively. At the end of fermentation, lower TVC was observed in NS and NSP. Final TVC of NNF, NS, NP and NSP were 7.99, 6.94, 7.75 and 6.92, respectively.

No detection of *S.* Typhimurium in NNF and NS. Therefore, the presence of *S.* Typhimurium in NP and NSP were only obtained from the inoculation. In NP (Figure 4.17C), the number of *S.* Typhimurium increased from 4.31 to 8.86 log CFU and remained constant until the end of fermentation time at 168 hours. Growth of *S.* Typhimurium in NSP (Figure 4.17D) was similar to NP at the initial of fermentation. The number of *S.* Typhimurium reached to 9.07 log CFU at 72 hours. No detection of *S.* Typhimurium was observed after 120 hours of fermentation time.

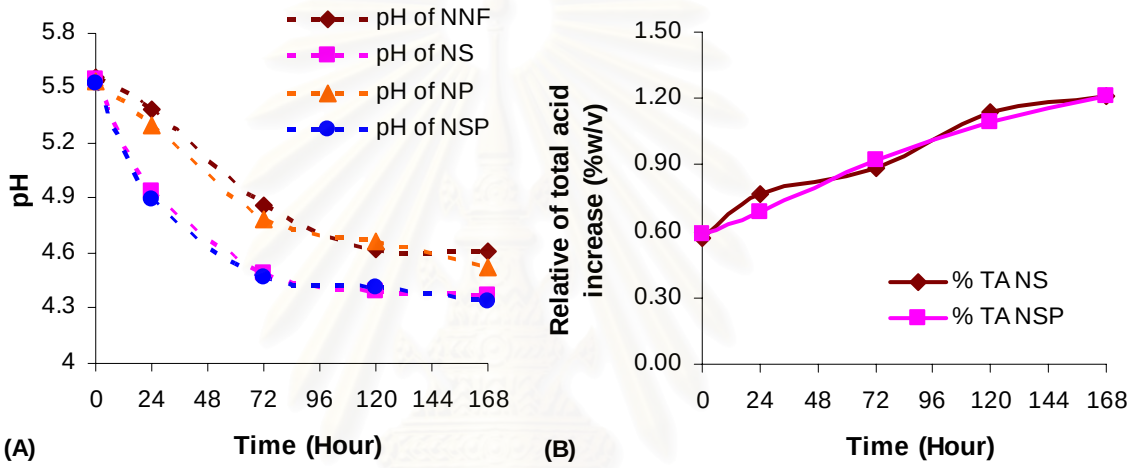


Figure 4.18 Changes in pH (A) and relative of total acid increase (%w/v) based on lactic acid production (B) of nham as a food model in the study of inhibitory effect on *S.* Typhimurium during fermentation.

Changes in pH and relative of total acid increase (%w/v) based of nham samples were shown in Figure 4.18A and B, respectively. At the end of fermentation, NS and NSP exhibited a lower pH than NNF and NP. Final pH of NNF, NS, NP and NSP were 4.61, 4.37, 4.52 and 4.34, respectively. Final relative of total acid increase of NS and NSP based on lactic acid production were 1.21 and 1.21, respectively. Changes in bacteria counts, pH and relative of total acid increase (%w/v) were shown in Table F22 (appendix F).

When inhibitory efficiency of *P. pentosaceus* NSL-132 against pathogenic bacteria in MRS broth and in food model were compared, the different results were observed. Complete growth inhibition of all pathogenic bacteria was obtained when co-cultured in MRS broth. In contrast to food model, when *P. pentosaceus* NSL-132 was applied as starter culture for nham preparation, it exhibited inhibitory effect on some strains of pathogenic bacteria including *S. aureus*, *B. cereus* and *S.* Typhimurium and complete growth inhibition in food model occurred lately than in MRS broth.

Major problem in the application of bacteriocin-producing starter strains in food fermentation related to antimicrobial efficacy which could be negatively influenced by various factors such as binding of the bacteriocins to food components (fat or protein particles) (Jung et al., 1992) and food additives (e.g. triglyceride oils, salt) (Leroy and De Vuyst, 1999a). In addition, proteases or other inhibitors, changes in solubility and charge and changes in the cell envelope of the target bacteria might be caused of inactivation (Degnan and Luchansky, 1992; Aesen et al., 2003). Moreover, meat which was a major raw material of nham provided more excellent nutrients and environment for growth of pathogenic and spoilage microorganisms than MRS broth that had some chemical compound acted as bactericidal substances. Therefore, many pathogenic bacteria were able to survive and reproduce well in meat more than complex medium like MRS.

In general, fermentation of nham involving successive growth of indigenous microorganisms were dominated by LAB. It had been playing an important role in food fermentation causing the sour taste, typical fermented aroma, firmness of texture together with a preservative effect resulting in an increase in shelf life of final products, since LAB was able to produce various antimicrobial substances including short-chain organic acids, carbon dioxide, hydrogen peroxide, diacetyl and bacteriocin (Rowan et al., 1998). The antimicrobial ability attributed to many LAB was primarily due to the production of organic acids as well as bacteriocins. Acid production contributes to both quality and safety in the production. A continuous decrease in pH was observed with increasing fermentation time causing in accumulation of organic acids that could prevent growth of sensitive pathogens.

From the results, the number of TVC and pathogenic bacteria counts decreased with pH decreased lower than 5.0. However, growth inhibition depended on not only the initial number of contamination but also sensitivity of each strain. Complete growth inhibition of *S. aureus* and *S.* Typhimurium were observed at pH lower than 4.37 and 4.47, respectively, while *B. cereus* was observed at pH lower 5.58. In contrast, survival of *L. monocytogenes* and *E. coli* were observed at this pH level. Valyasevi and Rolle (2002) described the growth of pathogenic bacteria such as *S. aureus, L. monocytogenes* and *Salmonella* sp. were inhibited by pH lower than 4.6. However, the ability of *L. monocytogenes* to survive in various environments was well known because it was capable to adapt for survival in environmental stressors such as acidic range pH, refrigerator temperature, low O_2 concentration, sodium chloride, heat, nitrite, and sorbate (McKellar, 1993; Myers and Martin, 1994; Phan-Thanh and Gormon, 1995). Although the final pH in nham product reached to 4.42 (Table F 19 in appendix F), growth of *L. monocytogenes* was found since it could grow at temperatures range from 0 to 45°C and pH 4.1–9.6 on foods for very long periods of time (Barbosa et al., 1994).

The rapid decrease in number of *B. cereus* was observed in both of nham inoculated with *P. pentosaceus* NSL13-2 and *B. cereus* (NSP) and nham inoculated with only *B. cereus* (NP). Complete growth inhibition of *B. cereus* was observed after 24 hours of fermentation time. In this case, growth inhibition might be caused by antagonistic activity from both of *P. pentosaceus* NSL13-2 and LAB flora obtained from raw materials. Acidic environment in nham was the one factor that resulted in non-survival of *B. cereus* because organic acid especially lactic acid could inhibit vegetative cell or spore germination. The similar result was found in other food models. Mikolajcik et al. (1973) reported that *B. cereus* increased initially at approximately the same rate in milk with or without *Streptococcus lactis*. When the acidity of the milk increased, vegetative *B. cereus* failed to survive but spore counts remained unchanged. However, spore germination, outgrowth, and vegetative cell multiplication were inhibited at pH 5.0. Moreover, *B. cereus* is an aerobic spore-forming bacteria, anaerobic condition in nham product could support to inhibit growth of this strain.

For Gram negative bacteria, *S.* Typhimurium exhibited acid sensitivity more than *E. coli*. At pH~4.4, no detection of *S.* Typhimurium was observed after 120 hours of fermentation time, while the number of *E. coli* remained at 3.67 log CFU. Survival of *E. coli* possibly occurred to acid adaptation that corresponded with the search of Kroll and Patchett (1992). However, higher decrease in number of *L. monocytogenes* and *E. coli* were observed in nham inoculated with *P. pentosaceus* NSL13-2 as starter culture (NSP) more than those of not inoculated with *P. pentosaceus* (NP). Therefore, the inhibitory effect on both bacterial pathogens caused by *P. pentosaceus* NSL13-2 could be implied.

Not only antibacterial substances produced from LAB but also some ingredients in nham were important factors that associated with growth inhibition of pathogenic strains. Garlic and curing salt were ingredients which had antagonistic activity against a number of pathogens. Because garlic had a major biologically compound namely as allicin and salt was associated with change in osmotic pressure of cell. Growth inhibitory effect of garlic had been found against different types of industrial and food spoilage yeasts as mentioned previously (Conner and Beuchat, 1984) and garlic was also found to inhibit food pathogens, such as *S. aureus*, *S.* Typhi, *E. coli* and *L. monocytogenes* (Kumar and Berwal, 1998).

Due to the presence of inhibitory efficiency of *P. pentosaceus* NSL13-2 which bacteriocin-producing strain against on certain pathogenic bacteria, the application of *P. pentosaceus* NSL13-2 as starter culture, using ingredients in appropriate proportions and good controlling during nham process were an alternative way to achieve high quality, consistency and safety in nham product.

CHAPTER V

CONCLUSION

A total of 107 LAB were isolated from 20 samples of nham provided from Suddhiluck Innofood Co.,Ltd. There were 42 rod-shaped and 65 cocci-shaped bacteria. All isolates were tested for antimicrobial activity by using agar spotted test and agar well diffusion method. For agar spotted test method, 65 LAB isolates were found to inhibit *S. aureus*, and then were tested with various indicator strains. There were LAB isolates 6, 5, 3 and 1 that found to exhibit inhibitory effect against *B. cereus*, *L. monocytogenes*, *E. coli* and *S.* Typhimurium, respectively. Whereas, only 34 isolates could perform inhibitory effect against *S. aureus*. In contrast, they could not inhibit *B. cereus*, *L. monocytogenes* and Gram-negative bacteria.

Strains NSL13-2, NSL1-4 and NSL5-2 which stably performed bacteriocin-producing activity were selected for identification. Based on carbohydrates fermentation patterns of API 50 CHL identification kit (BioMérieux, France), production of gas from glucose, growth in different conditions including at 10°C and 45°C, pH 4.4 and pH 9.6, 6.5% and 18% of NaCl concentration and 16S rDNA analysis were tested. Strains NSL13-2, NSL1-4 and NSL5-2 were identified as *Pediococcus pentosaceus*, *Weissella cibaria* and *Enterococcus gilvus*, respectively.

Inhibitory efficiency of 3 strains against pathogenic bacteria was investigated by co-culturing in MRS broth. The highest inhibitory efficiency was performed when 10^4 $CFUml^{-1}$pathogenic bacteria were co-cultured with 10^6 $CFUml^{-1}$ of *P. pentosaceus* NSL13-2. No detection of *S. aureus*, *L. monocytogenes* and *S.* Typhimurium after 12 hours, while *B. cereus* was not detected after 8 hours of incubation time. In all experiments, pH of MRS broth continuously decreased when fermentation times increased and bacteriocin production was found. From the results, *P. pentosaceus* NSL13-2 should applicably be used for study inhibitory efficiency against pathogenic strains in food model.

Moreover, influence of temperature, pH, NaCl concentration and incubation time on growth and bacteriocin activity of *P. pentosaceus* NSL13-2 were observed by using MRS broth as a culture medium. From the results, the highest number of *P. pentosaceus* NSL13-2 obtained when it was cultured in MRS broth that adjusted to pH 8.0 and incubated at 30°C for 24 hours. Whereas, the highest bacteriocin activity was obtained when cultured in MRS broth adding with 3% NaCl or adjusted to pH 6.0, 7.0 or 9.5. In addition, ranges of temperature from 25 to 37°C and incubation time at 12 and 24 hours were more favourable for bacteriocin production.

Inhibitory efficiency against pathogenic strains of *P. pentosaceus* NSL-132 in nham as a food model was observed. From the results, it exhibited inhibitory effect on some strains of pathogenic bacteria including *S. aureus*, *B. cereus* and *S.* Typhimurium. Whereas, decrease in number of *L. monocytogenes* and *E. coli* were observed.

No detection of *S. aureus* and *S.* Typhimurium were observed after 120 hours, while *B. cereus* was not detected after 24 hours in nham inoculated both of pathogenic bacteria and *P. pentosaceus* (NSP). The number of *L. monocytogenes* and *E. coli* in NSP showed the similar trend with NP. However, NSP showed the lower of *L. monocytogenes* and *E. coli* numbers than NP at complete fermentation time. In NSP, *L. monocytogenes* and *E. coli* were decreased to 2.26 and 3.23 log CFU, respectively.

In all experiments, changes of LAB and total viable counts (TVC) of NNF and NP showed the same trend, while NS performed the same trend with NSP. Decrease in the number of TVC in NS and NSP were generally higher than NNF and NP. Moreover, pH continuously decreased to the acidic range, while relative of total acid increase (%w/v) based on lactic acid production was increased during fermentation. Due to inhibitory efficiency of *P. pentosaceus* NSL13-2 against on certain pathogenic bacteria is present. The application of *P. pentosaceus* NSL13-2 as starter culture combining with using ingredients in appropriate proportions including good controlling during nham process will be the alternative way to achieve high quality, consistency and safeness in nham product.

REFERENCES

ภาษาไทย

พงษ์เทพ วิไลพันธ์. 2546. แบคเทอริโอซินจากแบคทีเรียกรดแล็กติกที่พบในปลาร้า. วิทยานิพนธ์ปริญญาดุษฎีบัณฑิต. เทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์.

อนงค์ เนตตะสูต. 2549. แหนม. กรุงเทพมหานคร: สำนักพิมพ์แม่บ้าน.

ภาษาอังกฤษ

Abee, T., Krockel, L. and Hill, C. 1995. Bacteriocins: modes of action and potential in food preservation and control of food poisoning. International of Food Microbiology 28: 169-185.

Aesen, I. M., Markussen, S., MØretrØ, T., Katla, T., Axelsson, L. and Naterstad, K. 2003. Interactions of the bacteriocins sakacin P and nisin with food constituents. International Journal of Food Microbiology 87: 35–43.

Afset, J. E., Bergh, K. and Bevanger, L. 2003. High prevalence of a typical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. Journal of Medical Microbiology 52: 1015-1019.

Andrews, W. H. and Hammack, T. S. 1998. *Salmonella*. FDA bacteriological analytical manual, 8th ed., rev. A. AOAC International, Gaithersburg, Md.

Anonymous. 2008. Knowthai[online]. Available from: http://www.becnews.com/backissue/k_knowthai/knowthai220447.html [2008, December 28]

Anonymous. 2009. *Bacillus cereus*[online]. Available from: http://wpcontent.answers.com/wikipedia/commons/thumb/0/02/Bacillus_cereus_01.png/240px-Bacillus_cereus_01.png[2009, February 3]

Anonymous. 2009. *Bacillus sp.*[Online]. Available from: http://atlas.medmicro.info/obrazek.php?id=1[2009, February 3]

Anonymous. 2009. Eosin Methylene Blue Agar (EMB)[online]. Available from: http://www.ams.cmu.ac.th/mt/clinmcrb/CMBwebsite/Price%20list_media.htm. [2009, March 5]

Anonymous. 2009. *Escherichia coli* on MacConkey agar[online]. Available from: http://www.nmconline.org/images/ecoli.jpg[2009, March 5]

Anonymous. 2009. Gram Stain Images[online]. Available from: http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Gram_Stain/Gram_stain_images/E_coli_2000_P7201172.jpg[2009, March 5]

Anonymous. 2009. *Listeria monocytogenes* in fish by Gram's staining[online]. Available from: http://hillagric.ernet.in/education/covas/vph/images/Lis.JPG [2009, February 3]

Anonymous. 2009. PALCAM Agar Base[online]. Available from: http://www.biotec.com/images/PalcamAgar.gif [2009, February 3]

Anonymous. 2009. *Samonella*[online]. Available from: http://www.podloza.pl/resources/files/870_1.jpg[2009, March 5]

Anonymous. 2009. *S. aureus*[online]. Available from: http://www.gslabs.com/images/saureus2.jpg[2009, March 4]

Anonymous. 2009. Staphylococcus aureus isolation with Lab M Baird-Parker medium[online]. Available from: http://www.labm.com/images/news_press_releases/t54_1.jpg[2009, March 2]

Anonymous. 2009. *S. aureus* The media Blood agar[online]. Available from: http://www.arabslab.com/vb/showthread.php?t=8567&page=4[2009, March 4]

AOAC. 2000. Official Methods of Analysis. In: 17th ed. Gaithersburg, Maryland: Association of Official Analytical Chemists.

Axelsson, L. T., Chung, T. C., Dobrogosz, W. J. and Lindgren, S. E. 1989. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. Microbial in Ecology Health and Disease 2: 131– 136.

Axelsson, H. 1998. Lactic acid bacteria: Classification and Physiology. In S. Saminen and A. von Wright (eds.), Lactic acid bacteria : Microbiology and functions aspect, 2nd ed, pp. 1-72. New York: Marcel Dekker, Inc.

Ayres, J. C. 1995. Microbiological implications in the handling, slaughtering, and dressing of meat animals. Advanced Food Research 6: 109-161.

Barbosa, W. B., Cabedo, L., WederWederquist, H. J., Sofos, J. N. and Schmidt, G. R. 1994. Growth variation among species and strains of *Listeria monocytogenes*. Journal of Food Protection 57 :765–769.

Bayane, A., Roblian, D., Dauphin, R. D., Destain, J., Diawara, B. and Thonart, P. 2006. Assessment of the phylological and biochemical characterization of a Lactic acid bacterium isolated from chicken faeces in sahelian region. African Journal of Biotechnology 5(8): 629-634.

Becker, H., Schaller, G., Von Wiese, W. and Terpian, G. 1994. *Bacillus cereus* in infant foods and dried milk products. International Journal of Food Microbiology 23: 1–15.

Belfiore, C. Castellano, P. and Vignolo, G. 2007. Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators. Food Microbiology 24: 223-229.

Benjakul, S., Seymour, T. A., Morissey, M. T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. Journal of Food Science 62: 729-733.

Benkerroum, N., Daoudi, A., Hamraoui, T., Ghalfi, H., Thiry, C., Duroy, M., Evrart, P., Roblain, D. and Thonart, P. 2005. Lyophilized preparations of bacteriocinogenic *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis* as potential protective adjuncts to control *Listeria monocytogenes* in dry-fermented sausages. Journal of Applied Microbiology 98: 56-63.

Bennik, M. H. J., Van Overbeek, W., Smid, E. J. and Gorris, L. G. M. 1999. Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. Letters in Applied Microbiology 28: 226-232.

Bhan, M. K., Bhandari, N., Sazawal, S., Clemens, J., Raj, P., Levine, M. M. and Kaper, J. B. 1989b. Descriptive epidemiology of persistent diarrhea among young children in rural northern India. Bull World Health Organ 67: 281-288.

Bhunia, A. K., Johnson, M. C. and Ray, B. 1988. Purification, characterisation and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. Journal of Applied Bacteriology 65: 261–268.

Blom, H. and Mortvedt, C. 1991. Anti-microbial substances produced by food-associated microorganisms. Biochemical Society Transactions 19: 694–698.

Bredholt, S. and Nesbakken, T. 1999. Protective cultures inhibit growth of *Listeria monocytogenes* and *Escherichia coli* o157: H7 in cooked, vacuum and gas-packaged meat. International Journal of Food Microbiology 53: 43-52.

Bredholt, S., Nesbakken, T. and Holck, A. 1999. Protective cultures inhibit growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in cooked, sliced, vacuum and gas-packaged meat. International Journal of Food Microbiology 53: 43-52

Bredholt, S., Nesbakken, T., and Holck, A. 2001. Industrial application of an antilisterial strain of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum-packaged meats. International Journal of Food Microbiology 66: 191-196.

Browning, N. G., Botha, J., Sacho, H. and Moore, P. J. 1990. *Escherichia coli* O157:H7 haemorrhagic colitis. Report of the first South African case. South African Journal of Surgery 28: 28–29.

Buckenhüskes, H. J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiology Reviews 12: 253–271.

Budde, B. B., Hornbæk, T., Jacobsen, T., Barkholt, V. and Koch, A. G. 2003. *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments. International Journal of Food Microbiology 83: 171-184.

Byczkowski, J. and Gessner, T. 1988. Biological role of superoxide ion-radical. International Journal of Biochemistry 20: 569–580.

Casman, E. P. 1965. Staphylococcal enterotoxin. Ann. NY Acad. Sci 128: 124–131.

Chart, H. 2002. *Escherichia*. In D. Greenwood, R. C. B. Slack and J. F. Peutherer (ed.), Medical Microbiology A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control, 16th ed, pp. 264-265. London: Churchill Livingstone.

Chen, H. D. and Frankel, G. 2005. Enteropathogenic *Escherichia coli* unraveling pathogenesis. FEMS Microbiology Reviews 29: 83-98.

Chung, T. C., Axelsson, L. T., Lindgren, S. E. and Dobrogosz, W. J. 1989. In vitro studies on reuterin synthesis by *Lactobacillus reuteri*. Microbial in Ecology Health and Disease 2: 137–144.

Cogan, T. M. and Hill, C. 1993. Cheese starter cultures. In P.F. Fox (Ed.), Cheese: Chemistry, Physics and Microbiology, vol. 1, 2nd ed, pp. 193–255. London: Chapman and Hall.

Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S. and Jones, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus *Carnobacterium*. International.Journal of Systematic Bacteriology 37: 310-316.

Collins, M. D., Ash, C., Farrow, J. A. E., Wallbanks, S. and Williams, A. M. 1989. 16S ribosomal ribonucleic acid sequence analyses of Lactococci and related taxa. Description of *Vagococcus flucialis* gen. nov.. sp. nov. Journal of Applied Bacteriology 67: 453-460.

Collins, M. D., Williams, A. M. and Wallbanks, S. 1990. The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. FEMS Microbiology Letters 70: 255-262.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. and Wallbanks, S. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiology Letters 77: 5-12.

Collins, M. D., Samelis, J., Metaxopoulus, J. and Wallbanks, S. 1993. Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissiella* for the *Leuconostoc paramesenteroides* group of species. Journal of Applied Bacteriology 75: 595-603.

Conner, D. E. and Beuchat, R. 1984. Effects of essential oils from plants on growth of food spoilage yeasts. Journal of Food Science 49: 429–434.

Daeschel, M. A., Andersson, R. E. and Fleming, H. P. 1987. Microbial ecology of fermenting plant materials. FEMS Microbiology Reviews 46: 357-367.

Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technology 43(1): 164-167.

Daeschel, M. A., Jung, D. and Watson, B. T. 1991. Controlling wine malolactic fermentation with nisin and nisin-resistant strains of *Leuconostoc oenos*. Applied and Environmental Microbiology 57: 601-603

Daly, C., Sandine, W. E. and Elliker, P. R. 1970. Interactions of food starter cultures and food-borne pathogens: *Streptococcus diacetylactis* versus food pathogens. Journal of Milk and Food Technology 35: 349-357.

Daly, C. and Davis, R. 1998. The biotechnology of lactic acid bacteria with emphasis on applications in food safety and human health. Agriculture Food Science in Finland 7: 251–265.

Darnton N. C., Turner, L., Rojevsky, S. and Berg H. C. 2007. On torque and tumbling in swimming *Escherichia coli*. Journal of Bacteriology 189(5):1756-1764.

Dawson, P. L., Hirt, D. E., Rieck, J. R., Acton, J. C. and Sotthibandhu, A. 2003. Nisin release from films is affected by both protein type and film-forming method. Food Research International 36: 959-968.

Degnan, A. and Luchansky, J. 1992. Influence of beef tallow and muscle on the antilisterial activity of pediocin AcH and liposome-encapsulated pediocin AcH. Journal of Food Protection 55: 552–554.

Deibel , R. H. and Niven Jr, C. F. 1961. The genus *Pediococcus*. In B. J. B. Wood and W. H. Holzapfel (eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 125-131. Glasgow, UK: Chapman and Hall.

Dellagio, F. L., Dicks, M. T. and Torriani , S. 1995. The genus *Leuconostoc*. In B. J. B. Wood and W. H. Holzapfel (eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 235-278. Glasgow, UK: Chapman and Hall.

De Martinis, E. C. P. and Freitas, F. Z. 2003. Screening of lactic acid bacteria from Brazilian meats for bacteriocin formation. Food Control 14: 197-200.

Denny, C. B., Humber, J. Y. and Bohrer, C. W. 1971. Effect of toxin concentration on the heat inactivation of staphylococcal enterotoxin A in beef bouillon and in phosphate buffer. Applied Microbiology 21: 1,064–1,066.

Devriese, L. A. and Pot, B. 1995. The genus *Enterococcus*. In B. J. B. Wood and W. H. Holzapfel (editors), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 327-367. London: Blackie Academic.

Dicks, L. M. T., Dellaglio, F. and Collins, M. D. 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. International.Journal of Systematic Bacteriology 45: 395-397.

Dobrogosz, W. J., Casas, I. A., Pagano, G. A., Talarico, T. L., Sjöberg, B. M. and Karlsson, M. 1989. *Lactobacillus reuteri* and the enteric microbiota. In R. Gruff, T. Midtvedt and E. Norin (Eds.), The Regulatory and Protective Role of the Normal Microflora, pp. 283–292. New York: Stockton Press.

Dupont, H. L., Formal, S. B., Hornick, R. B., Snyder, M. J., Libonati, J. P., Sheahan, D. G., LaBrec, E. H. and Kalas, J. P. 1971. Pathogenesis of *Escherichia coli* diarrhea. The New England Journal of Medicine 285: 1-9.

Eklund, T. 1984. The effect of carbon dioxide on bacterial growth and on uptake processes in the bacterial membrane vesicles. International Journal of Food Microbiology 1: 179–185.

Evans, D. G., Evans, D. J. Jr. and DuPont, H. L. 1997. Virulence factors of enterotoxigenic *Escherichia coli*. Journal of infectious Diseases 136(Suppl): S118-237.

Farber, J. M. 1991. Microbiological aspects of modified-atmosphere packaging technology—a review. Journal of Food Protection 54: 58–70.

Foster, E. M. 1969. The problem of salmonellae in foods. Food Technology 23: 1,178

Franz, C. M., Holzapfel, W. H. and Stiles, M. E. 1999. Enterococci at the crossroads of food safety. International Journal of Food Microbiology 47: 1-24.

Franzmann, P. D., Höpfl, P., Weiss, N. and Tindall, B. J. 1991. Psychrotrophic, lactic acid producing bacteria from anoxic waters in Ace Lake, Antarctica; *C. funditum* sp. nov. and *C. alterfunditum* sp. nov. Arch. Microbiology 156: 255-262.

Galane, P. M. and Le Roux, M. 2001. Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarrhoeal diseases. Journal of Health Population and Nutrition 19(1): 31–37.

Gänzle, M., Weber, S. and Hammes, W. 1999. Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. International Journal of Food Microbiology 46: 207–217.

Garmendia, J., Frankel, G. and Crepin, V. F. 2005. Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. Infection and Immunity 73: 2573—2585.

Garneau, S., Martin, N. I. and Vederas, J. C. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie 84: 577-592.

Garvie, E. I. 1986a. Genus *Leuconostoc* van Tieghem 1878. In In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (editors), Bergey's manual of systematic bacteriology, pp. 1071-1075. Baltimore, MD: Williams and Wilkins.

Garvie, E. I. 1986b. Genus *Pediococcus* Claussen 1903. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (editors), Bergey's manual of systematic bacteriology, pp. 1075-1079. Baltimore, MD: Williams and Wilkins.

Gilbert, R. J. 1979. *Bacillus cereus* gastroenteritis. In H. Reimann and F. L. Bryan (ed.), Food-borne infections and intoxications, 2nd ed., p. 495-518. New York: Academic Press.

Gilligan, P. H., Brown, L. and Berman, R. E. 1983. Differentiation of *Clostridium difficile* toxin from *Clostridium botulinum* toxin by the mouse lethality test. Applied Environmental Microbiology 45: 347–349.

Gonzalez, C. F. and Kunka, B. S. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. Applied Environmental Microbiology 53: 2,534–2,538.

Gorris, L. G. M. 1997. Bacteriocins: potential applications in food preservation, pp. 79-86. In Food preservation by combined processed. Final report. FLAIR Concerted Action No. 7 Subgroup B.UE. DGXII.

Granum, P. E. 1994. *Bacillus cereus* and its toxins. Journal of Applied Bacteriology Symposium Supplement 76: 61S-66S.

Gravani, R. B. 1984. Salmonellosis-*Salmonella* food poisoning. Journal of Dairy Science 75: 2,327-2,343.

Gray, M. L. and Killinger, A. H. 1966. *Listeria monocytogenes* and listeric infection. Bacteriological Reviews 30: 309-382.

Gross, E. and Morell, J. L. 1967. The presence of dehydroalanine in the antibiotic nisin and its relationship to activity. Journal of the American Chemical Society 89: 2791.

Hammes, W. P., Bantleon, A. and Min, S. 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiology Reveiws 87: 165-173.

Hammes, W. P., Weiss, N. and Holzapfel. W. 1991. The genera *Lactobacillus* and *Carnobacterium*. In A. Balous, H. G. Triiper, M. Dworkin, W. Harder and K.-H. Schleifer (editors), The Prokaryotes, Vol. 2, 2nd edition, pp. 1535-1594. New York: Springer-Verlag.

Hardie, J. M. and Whiley, R. A. 1995. The genus *Streptococcus.* In B. J. B. Wood and W. H. Holzapfel (eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 55-125. Glasgow, UK: Chapman & Hall.

Heijne, G. V. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Research 14: 4,683-4,690.

Heijne, G. V. 1988. Transcending the impenetrable: how proteins come to terms with membranes. Biochem. Biophys. Acta 947, 307-333.

Hernandez, F. J., Goyache, J., Orden, J. A., Blanco, J. L., Domenech, A. and Suarez, G. et al. 1993. Repair and enterotoxin synthesis by *Staphylococcus aureus* after thermal shock. Applied Environmental Microbiology 59: 1,515–1,519.

Hitchins, A. D., Feng, P., Watkins, W. D., Rippey, S. R. and Chandler, L. A. 1998. *Escherichia coli* and the coliform bacteria. FDA bacteriological analytical manual, 8th ed., rev. A. AOAC International, Gaithersburg, Md.

Hitchins, A. D. 2000. *Listeria monocytogenes*. FDA bacteriological analytical manual, 8th ed., rev. A. AOAC International, Gaithersburg, Md.

Holzapfel, R. W., Geisen, R. and Schillinger, U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology 24: 343-362.

Holzapfel, W. H. and Stiles, M. E. 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology 36: 1-29.

Horn, N., Swindell, S., Dodd, H. and Gasson, M. 1991. Nisin biosynthesis genes are encoded by a novel conjugative transposon. Molecular and General Genetics 228: 129-135.

Jack, R. W., Tagg, J. R. and Ray, B. 1995. Bacteriocins of Gram-positive bacteria. Microbiological Reviews 59: 171-200.

Jay, J. M. 1982. Antimicrobial properties of diacetyl. Applied and Environmental Microbiology 44: 525–532.

Jay, J. M. 1986. Modern food microbiology. New York: Van Nostrand Reinhold.

Jay, J. M. 1996. Microorganisms in fresh ground meats: the relative safety of products with low versus high numbers. Meat Science 43: S59-S65.

Jay, J. M. 1997. Modern food microbiology, 5th ed. New York: Chapman and Hall.

Joerger, M. C. and Klaenhammer, T. R. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. Journal of Bacteriology 167: 439 – 446.

Jones, D. 1978. Composition and differentiation of the genus *Streptococcus*. In F. A. Skinner and L. B. Quesnel (editors), Streptococci, pp. 1-49. London: Academic Press.

Jung, D. S., Bodyfelt, F. and Daeschel, M. 1992. Influence of fat and emulsifiers on the efficiency of nisin in inhibiting *Listeria monocytogenes* in fluid milk. Journal of Dairy Science 75: 387–393.

Juven, B. J., Barefoot, S. F., Pierson, M. D., McCaskill, L. H. and Smith, B. 1998. Growth and survival of *Listeria monocytogenes* in vacuum-packaged ground beef inoculated with *Lactobacillus alimentarius* FloraCarn L-2. Journal of Food Protection 61: 551-556.

Kaper, J. B. 1998. Enterohemorrhagic *Escherichia coli*. Current Opinion in Microbiology 1: 103–108.

Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70: 337–349.

Kong, S. and Davison, A. J. 1980. The role of interactions between O_2, H_2, OH^-, e^- and O^{2-} in free radical damage to biological systems. Archives of Biochemistry and Biophysics 204: 18–29.

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes and Infection 2(2): 189–198.

Kumar, M. and Berwal, J. S. 1998. Sensitivity of food pathogens to garlic (*Allium sativum*). Journal of Applied Microbiology 84: 213–215.

Kramer, J. M. and Gilbert, R. J. 1989. *Bacillus cereus* and other *Bacillus* species. In M.P. Doyle (Ed.), Foodborne Bacterial Pathogens, pp. 21– 70. New York: Marcel Dekker.

Kroll, R. G. and Patchett, R. A. 1992. Induced acid tolerance in *Listeria monocytogenes* . Letters in Applied Microbiology 14: 224–227.

Kim, W. S., Hall, R. J., Dunn, N. W. 1997. The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. Applied Microbiology and Biotechnology 50: 429–433.

Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. Journal of Infectious Diseases 155: 377-389.

Leroy, F. and De Vuyst, L. 1999. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin Sakacin K. Applied and Environmental Microbiology 65: 974-981.

Leroy, F. and De Vuyst, L. 1999a. The presence of salt and a curing agent reduces bacteriocin production of *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Applied and Environmental Microbiology 65: 5350–5356.

Leroy, F. and De Vuyst, L. 2001. Growth of the bacteriocin-producing *Lactobacillus sakei* CTC 494 in MRS broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for the growth of lactic acid bacteria. Applied and Environmental Microbiology 67:4470–4473.

Leroy, F., Vankrunkelsven, S., De Greef, J. and De Vuyst, L. 2003. The stimulating effect of a harsh environment on the bacteriocin activity by *Enterococcus faecium* RZS C5 and dependency on the environmental stress factor used. International Journal of Food Microbiology 83: 27–38.

Lewus, C. B., Kaiser, A. and Montville, T. J. 1991. Inhibition of food-borne pathogens by bacteriocins from lactic acid bacteria isolated from meat. Applied and Environmental Microbiology 57(6): 1683-1688.

Liepe, H. U. 1983. Starter cultures in meat production. In H. J. Rehm and G. Reed (ed.), Biotechnology, vol. 5, pp. 399-424. Federal Republic of Germany: Verlag Chemie and Weinheim.

Lindgren, S. E. and Dobrogosz, W. J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Reviews 87: 149–163.

Lund, T., and Granum, P. E. 1997. Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. Microbiology 143: 3,329–3,333.

Magwira, C. A., Gashe, B. A. and Collison, E. K. 2005. Prevalence and antibiotic resistance profiles of *Escherichia coli* O157:H7 in beef products from retail outlets in Gaborone, Botswana. Journal of Food Protection 68 (2): 403–406.

Marrug, J. D. 1991. Bacteriocins, their role in developing natural products. Food Biotechnology 5: 305-312.

Mataragas, M., Skandamis, P. N. and Drosinos, E. H. 2008. Risk profiles of pork and poultry meat and risk ratings of various pathogen/product combinations. International Journal of Food Microbiology 126: 1-12.

Mayr-Harting, A., Hedges, A. J. and Berkeley, R. C. W. 1972. Methods for studying bacteriocins. In J. R. Norris and D. W. Ribbon(eds.), Methods in Microbiology(7a), pp. 313-342. New York: Academic Press.

Måyrå-Måkinen, A. and Suomalainen, T. 1995. *Lactobacillus casei* spp. *rhamnosus*, bacterial preparations comprising said strain and use of said strain and preparations for the controlling of yeast and moulds. U.S. Patent US 5: 378 458.

McKellar, R. C. 1993. Effect of preservatives and growth factors on secretion of listeriolysin O by *Listeria monocytogenes*. Journal of Food Protection 56: 380–384.

Mead, G. C., Norris, A. P. and Bratchell, N. 1989. Differentation of *Staphylococcus aureus* from freshly slaughtered poultry and strains ‘‘endemic’’ to processing plants by biochemical and physiological tests. Journal of Applied Bacteriology 66: 153–159.

Mikolajcik, E. M., Kearney, J. W. and Kristoffersen, T. 1973. Fate of *Bacillus cereus* in cultured and direct acidified skim milk and cheddar cheese. Journal of Milk and Food Technology 36:317-320.

Miller, K. W., Ray, P., Steinmetz, T. Hanekamp, B. and Ray, B. 2005. Gene organization and sequences of pediocin AcH/PA-1 production operons in *Pediococcus* and *Lactobacillus* plasmids. Letters in Applied Microbiology 40: 56-62.

Minor, L. L. E. 1984. *Sallmonella.* In J. G. Holt (ed), Bergey’s Manual of Systematic Bacteriology, 9th ed., p. 427. Baltimore, MD: Williams and Wilkins.

Montville, T. J., Winkowski, K. and Ludescher, R. D. 1995. Models and mechanisms for bacteriocin action and application. International Dairy Journal 5: 797-814.

Myers, E. R. and Martin, S. E. 1994. Virulence of *Listeria monocytogenes* propagated in NaCl containing media at 4, 25 and 37°C. Journal of Food Protection 57: 475–478.

Nataro, J. P., Kaper, J. B., Robins-Browne, R., Prado, V., Vial, P. and Levine, M. M. 1987. Patterns of adherence of diarrheagenic *Escherichia* coli toHEp-2 cells. Pediatric Infectious Disease Journal 6: 829–831.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews 11: 142—201.

Nel, H. A., Bauer, R., Vandamme, E. J. and Dicks, L. M. T. 2001. Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. Journal of Applied Microbiology 91: 1131-1138.

Nes, I. F., Diep, D. B., Havarstein, L. S., Brurberg, M. B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antoine van Leeuwenhoek 70: 113–128.

Noonpakdee, W., Santivarangkna, C. Jumriangrit, P., Sonomoto, K. and Panyim, S. 2003. Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a tradition Thal fermented sausage. International Journal of Food Microbiology 81: 137-145.

Notermans, S., Dufrenne, J. and Van Leeuwen, W. J. 1982. Contamination of broiler chickens by *Staphylococcus aureus* during processing, incidence and origin. Journal of Applied Bacteriology 52: 275–280.

Notermans, S., Van Leeuwen, W. J. and Rost, J. A. 1983. *Staphylococcus aureus* indigenous to poultry processing plants; persistence, enterotoxigenicity and biochemical characteristics. In C. Lahellec, F. H. Richard and P. Colin (Eds.), Quality of Poultry Meat. Proceedings of sixth European Symposium on Poultry Meat Quality, pp. 255-266. France: Ploufragan.

Obi, C. L., Potgieter, N., Bessong, P. O., Igumbor, E. O. and Green, E. 2004. Gene encoding virulence makers among *Escherichia coli* isolates from diarrheic stools samples and river sources in rural Venda communities of South Africa. Water SA 30(1): 37–42.

Ogunbanwo, S. T., Sanni, A. I. and Onilude, A. A. 2003. Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. African Journal of Biotechnology 2(7): 179-184.

Osmanağaoğlu, Ö., Beyatli, Y. and Gündüz, U. 2001. Isolation and characterization of pediocin-producing *Pediococcus pentosaceus* Pep1 from vacuum-packed sausages. Turkish Journal of Biology 25: 133-143.

Parente, E. and Ricciardi, A. 1994. Influence of pH on the production of enterocin 1146 during batch fermentation. Letter in Applied Microbiology 19: 12–15.

Parker, M. T. 1978. The pattern of streptococcal disease in man. In F. A. Skinner and L. B. Quesnel (eds.), Streptococci. Soc. Appl. Bacteriol. Symp Series, No.7, pp. 71-106. London: Academic Press.

Paul M., Tsukamoto, T., Ghosh, A. R., Bhattacharya, S. K., Manna, B., Chakrabarti, S., Nair, G. B., Sack, D. A., Sen, D. and Takeda, Y. 1994. The significance of enteroaggregative *Escherichia coli* in the etiology hospitalized diarhea in Calcuta, India and the demonstration of a new honey-combed pattern of aggregative adherence. FEMS Microbiology Letters 117: 319-325.

Peng, H., Ford, V., Frampton, E. W., Restaino, L., Shelef, L. A. and Spitz, H. 2001. Isolation and enumeration of *Bacillus cereus* from foods on a novel chromogenic plating medium. Food Microbiology 18: 231-238.

Petchsing, U. and Woodburn, M. J. 1990. *Staphylococcus aureus* and *Escherichia coli* in nham (Thai-style fermented pork sausage). International Journal of Food Microbiology 10: 183-192.

Phan-Thanh, L. and Gormon, T. 1995. Analysis of heat and cold shock proteins in *Listeria* by two-dimensional electrophoresis. Electrophoresis 16: 444–450.

Phithakpol, B., Varanyanond, W., Reunmaneepaitoon, S. and Wood, H. 1995. The traditional fermented foods of Thailand. Bangkok, Thailand: Institute of Food Research and Product Development, Kasetsart University.

Piard, J. C., Delorme, F., Giraffa, G., Commissaire, J. and Desmazeaud, M. 1990. Evidence for a bacteriocin produced by *Lactococcus lactis* CNRZ 481. Netherlands Milk and Dairy Journal 44: 143 – 158.

Piard, J. C. and Desmazeaud, M. 1991. Inhibiting factors produced by lactic acid bacteria: 1. Oxygen metabolites and catabolism end products. Lait 71: 525–541.

Piard, J. C. and Desmazeaud, M. 1992. Inhibiting factors produced by lactic acid bacteria: 2. Bacteriocins and other antibacterial substances. Lait 72: 113–142.

Podolak, P. K., Zayas, J. F., Kastner, C. L., and Fung, D. Y. C. 1996. Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef by application of organic acids. Journal of Food Protection 59: 370–373.

Ponce, A. G., Moreira, M. R., del Valle, C. E. and Roura, S. I. 2008. Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. LWT 41: 432-441.

Pot, B., Ludwig. W., Kersters, K. and Schleifer, K. H. 1994. Taxonomy of lactic acid bacteria. Microbiology, genetics and applications. In L. De Vuyst and E. J. Vandamme (editors), Bacteriocins of Lactic Acid Bacteria, pp. I3 -90. London: Chapman and Hall.

Riebroya, S., Benjakula, S. and Visessanguan, W. 2008. Properties and acceptability of Som-fug, a Thai fermented fish mince, inoculated with lactic acid bacteria starters. LWT 41: 569-580.

Roberts, D. 1982. Bacteria of public health significance. In M. H. Brown (ed.), Meat Microbiology, pp. 319-386. London: Applied Science Publishers Ltd.

Rowan, N. J., Anderson, J. G. and Smith, J. E. 1998. Potential infective and toxic microbiological hazards associated with the consumption of fermented food. In B. J. B. Wood (Ed.), Microbiology of Fermented Foods. London: Blackie Academic and Professional.

Ruhr, E. and Sahl H. G. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrobial Agents and Chemotherapy 27: 841-845.

Ryan, K. J. and Ray, C. G. 2004. Sherris Medical Microbiology. 4th ed. McGraw Hill. pp. 362–368.

Ryser, E. T. 1999. Incidence and behavior of *Listeria monocytogenes* in unfermented dairy products. In E. T. Ryser and E. H. Marth (ed.), *Listeria*, Listeriosis and Food Safety, pp. 359–409. New York: Marcel Dekker.

Schillinger, U., Geisen, R. and Holzapfel, W. H. 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends in Food Science and Technology 7: 158–164.

Schlech, W. F. 2000. Foodborne listeriosis. Clinical Infectious Diseases 31: 770–775.

Schleifer, K. H. and Stackebrandt, E. 1983. Molecular systematics of procaryotes. Annual Reveiw of Microbiology 37: 143-187.

Schleifer, K. H. and Kilpper-Bälz, R. 1987. The genus *Enterococcus*. In B. J. B. Wood and W. H. Holzapfel (eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 327-332. Glasgow, UK: Chapman and Hall.

Settanni, L., Valmorri, S., Suzzi, G. and Corsetti, Aldo. 2008. The role of environmental factors and medium composition on bacteriocin-like inhibitory substances (BLIS) production by *Enterococcus mundtii* strains. Food Microbiology 25: 722-728.

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

Simpson, W. J. and Taguchi, H. 1995. The genus *Pediococcus* with notes on the genera *Tetragenococcus* and *Aerococcus*. In B. J. B. Wood and W. H. Holzapfel(eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 126-164. Glasgow, UK: Chapman & Hall.

Snyder, J. D., Wells, J. G., Yashuk, J., Puhr, N. and Blake, P. A. 1984. Outbreak of invasive *Escherichia coli* gastroenteritis on a cruise ship. American Journal of Tropical Medicine and Hygiene 33: 281-284.

Srionnual, S., Yanagida, F., Lin, L. H., Hsiao, K. N. and Chen, Y. 2007. Weissellicin 110, a newly discovered bacteriocin from *Weissella cibaria* 110, isolated from Plaa-Som, a fermented fish product from Thailand. Applied and Environmental Microbiology 73: 2,247-2,250.

Steen, M. T., Chung, Y. J. and Hansen, J. N. 1991. Characterization of the nisin gene as part of a polycistronic operon in the chromosome of *Lactococcus lactis* ATCC 11454. Applied and Environmental Microbiology 57: 1181-1188.

Stevens, K. A., Sheldon, B. W., Klapes, N. A. and Klaenhammer, T. R. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Applied and Environmental Microbiology 57:3616-3615.

Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antoine van Leeuwenhoek 70: 331-345.

Stiles, M. E. and Holzapfel, W. H. 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology 36: 1-29.

Suwanjindat, D., Eamest, C. and Panbangreds, W. 2007. Screening of lactic acid bacteria for bacteriocins by microbiological and PCR methods. Biochemistry and Molecular Biology Education 35(5): 364-369

Tanasupawat, S. and Daengsubha, W. 1983. *Pediococcus* species and related bacteria found in fermented foods and related materials in Thailand. Journal of General and Applied Microbiology 29: 487–506.

Tanasupawat, S. and Komagata, K. 1995. Lactic acid bacteria in fermented foods in Thailand. World Journal of Microbiology and Biotechnology 11: 253– 256.

Tamarapu, S., McKillip, J. L. and Drake, M. 2001. Development of a multiplex Polymerase chain reaction assay for detection and differentation of *Staphylococcus aureus* in dairy products. Journal of Food Protection 64: 664–668.

Tauxe, R. V. 2002. Emerging foodborne pathogens. International Journal of Food Microbiology 78: 31–41.

Taylor, D. N., Echeverria, P., Pal, T., Sethabutr, O., Saiborisuth, S., Srichamorn, S., Rowe, B. and Cross, J. 1986. The role of *Shigella* spp., enteroinvasive *Escherichia coli* and other enteropathogens as causes of childhood dysentery in Thailand. Journal of Infectious Diseases 153: 1132-1138.

Teuber, M. 1995. The genus *Lactococcus*. In B. J. B. Wood and W. H. Holzapfel (eds.), The Lactic Acid Bacteria vol. 2: The Genera of Lactic Acid Bacteria, pp. 173-174. Glasgow, UK: Chapman and Hall.

Todar, K. 2009. Isolation and Identification of *Salmonella*[online]. Available from: http://bioinfo.bact.wisc.edu/themicrobialworld/Salmonella.XLD.jpg[2009, March 5]

Todar, K. 2009. *Salmonella* Nomenclature[online]. Available from: http://bioinfo.bact.wisc.edu/themicrobialworld/S.typhi.Gram.jpg[2009, March 5]

Tortorello, M. L. 2000. *Escherichia coli* O157:H7. In R. K. Robinson, C. A. Batt and P. D. Patel (Eds.), Encyclopedia of Food Microbiology, Vol. 1, pp. 646–652. London: Academic Press.

Turnbull, P. C. B., Kramer, J. M., Jorgensen, K., Gilbert, R. J. and Melling, J. 1979. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. American Journal of Clinical Nutrition 32: 219–228.

Tsch.ape, H. and Fruth, A. 2001. Enterohemorrhagic *Escherichia coli* in Emerging Bacterial Pathogens. Contribution of Microbiology 8: 1–11.

Vallance, B. A. and Finlay, B. B. 2000. Exploitation of host cells by enteropathogenic *Escherichia coli*. Proceedings of the National Academy of Sciences USA 97: 8799-8806.

Valyasevi, R., Jungsiriwat, P., Smitinont, T., Praphailong, W. and Chowalitnitithum, C. 2001. Improvement of starter culture for nham fermentation. Final report submitted to National Center for the Genetic Engineering and Biotechnology. National Science and Technology Development Agency.

Valyasevi, R. and Rolle, R. S. 2002. An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: scope for their improvement. International Journal of Food Microbiology 75: 231– 239.

Vandamme, P., Pot, B., Gllis, M., de Vos, P., Kersters, K. and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiology Reveiws 60: 407-438.

Van den Bussche, R. A., Lyon, J. D. and Bohach, G. A. 1993. Molecular evolution of the staphylococcal and streptococcal pyrogenic toxin gene family. Molecular Phylogenetics and Evolution 2: 281–292.

Varnam, A. H. 1995. Sutherland, J. Cooked cured meats. In A. H. Varnam, J. P. Sutherland (eds), Meat and Meat Products, Technology, Chemistry and Microbiology, pp. 298-313. London: Chapman & Hall.

Vermeiren, L., Devlieghere, F. and Debevere, J. 2006. Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham. International Journal of Food Microbiology 108: 68-77.

Vermeiren, L., Devlieghere, F., Vandekinderen, I. and Debevere, J. 2006. The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiology 23: 511-518.

Vignolo, G., Fadda, S., de Kairuz, M. N., de Ruiz Holgado, A. A. P. and Oliver, G. 1996. Control of *Listeria monocytogenes* in ground beef by 'Lactocin 705', a bacteriocin produced by *Lactobacillus casei* CRL 705. International Journal of Food Microbiology 29: 397–402.f

Visessanguan, W., Benjakul, S., Smitinont, T., Kittikun, C., Thepkasikul, P. and Panya, A. 2006. Changes in microbiological, biochemical and physico-chemical properties of Nham inoculated with different inoculum levels of *Lactobacillus curvatus*. LWT 39: 814-826.

Von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Research 14: 4683-4690.

Von Heijne, G. 1988. Transcending the impenetrable: how proteins come to terms with membranes. Biochem. Biophys. Acta 947: 307-333.

Wallbanks, S., Martinez-Murcia, A. J., Fryer, J. L., Phillips, B. A. and Collins, M. D. 1990. 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmonlnarum* sp. nov. International Journal of Systematic Bacteriology 40: 224-230.

Wieneke, A. A., Roberts, D. and Gilbert, R. J. 1993. Staphylococcal food poisoning in the United Kingdom. Epidemiology and Infection 110: 519– 531.

Wiriyacharee, P., Brooks, J. D., Earle, M. D. and Page, G. 1990. The improvement of a traditional Thai fermented pork sausage by used of mixed starter culture. In Fermentation technologies, Industrial applications conference, Massey University. New Zealand: Palmerston North.

APPENDICES

Appendix A

Media Preparation

1. Baird – Parker Agar Base

Pancreatic Digest of Casein	10.0	g
Beef Extract	5.0	g
Yeast Extract	1.0	g
Glycine	12.0	g
Sodium Pyruvate	10.0	g
Lithium Chloride	5.0	g
Agar	20.0	g
Egg Yolk Tellurite Enrichment	50.0	ml

Procedure

All of ingredients excepted Egg Yolk Tellurite Enrichment were dissolved in 950 ml of distilled water. Mix it thoroughly and adjust pH to 6.9 $\pm$ 0.1. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes. Cool to 45 – 50°C and aseptically add 50 ml of Egg Yolk Tellurite Solution.

2. Bismuth Sulfite Agar

Beef Extract	5.0	g
Peptone	10.0	g
Dextrose	5.0	g
Disodium Phosphate	4.0	g
Ferrous Sulfate	0.3	g
Bismuth Sulfite Indicator	8.0	g
Agar	20.0	g
Brilliant Green	25.0	mg

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.7±0.2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder (Do not autoclave).

3. Eosin Methylene Blue Agar, Modified,Holt-Harris and Teague (EMB)

Pancreatic Digest of Gelatin	10.0	g
Lactose	5.0	g
Sucrose	5.0	g
Dipotassium Phosphate	2.0	g
Eosin Y	0.4	g
Methylene Blue	65.0	mg
Agar	13.5	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.2 ± 0.2. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes.

4. Hektoen Enteric agar

Proteose Peptone	12.0	g
Yeast Extract	3.0	g
Bile Salts No.3	9.0	g
Lactose	12.0	g
Saccharose	12.0	g
Salicin	2.0	g
Sodium Chloride	5.0	g
Sodium Thiosulfate	5.0	g
Ferric Ammonium Citrate	1.5	g

Agar	14.0	g
Bromthymol Blue	65.0	mg
Acid Fuchsin	0.1	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.5±0.2. Heat with frequent agitation and boil to completely dissolve the powder (Do not overheat and autoclave).

5. Lactobacilli MRS Broth

Proteose Peptone No. 3	10.0	g
Beef Extract	10.0	g
Yeast Extract	5.0	g
Dextrose	20.0	g
Polysorbate 80	1.0	g
Ammonium Citrate	2.0	g
Sodium Acetate	5.0	g
Magnesium Sulfate	0.1	g
Manganese Sulfate	0.05	g
Dipotassium Phosphate	2.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and autoclave at 110°C for 20 minutes.

6. Lactose broth

Beef Extract	3.0	g
Peptone	5.0	g
Lactose	5.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly. Adjust pH to 6.9±0.2. Autoclave at 121°C for 15 minutes.

7. Lysine Iron Agar

Peptone	5.0	g
Yeast Extract	3.0	g
Dextrose	1.0	g
L-Lysine HCl	10.0	g
Ferric Ammonium Citrate	0.5	g
Sodium Thiosulfate	0.04	g
Bromcresol Purple	0.02	g
Agar	15.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 6.7±0.2. Heat for 1 minute to completely dissolve. Dispense into tubes and autoclave at 121°C for 15 minutes. Cool in a slanted position so that deep butts are formed.

8. Mannitol Salt Agar (MSA)

Proteose Peptone No. 3	10.0	g
Beef Extract	1.0	g
D-Mannitol	10.0	g
Sodium Chloride	75.0	g
Agar	15.0	g
Phenol Red	25.0	mg

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.4 $\pm$ 0.2. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes.

9. Motility Medium

Tryptone	10.0	g
Sodium Chloride	5.0	g
Agar	5.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly. Heat in order to completely dissolve. Dispense into tubes and autoclave at 121°C for 15 minutes.

10. MRS Agar

Procedure

Consists of the same ingredients of Lactobacilli MRS Broth. 16 g of agar was added in 1 L of MRS Broth. Heat in order to completely dissolve and autoclave at 110°C for 20 minutes.

11. MRS Agar with 0.04% bromocresol purple

Procedure

Consists of the same ingredients of Lactobacilli MRS Broth. 0.4 g of bromocresol purple was added in 1 L of MRS Broth. Adjust pH to 7.0. 16 g of agar was added. Heat in order to completely dissolve and autoclave at 110°C for 20 minutes.

12. MR-VP Medium

Buffered Peptone	7.0	g
Dipotassium Phosphate	5.0	g
Dextrose	5.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly. Adjust pH to 6.9±0.2. Dispense into tubes and autoclave at 121°C for 15 minutes.

13. MYP Agar Antimicrobic Vial P

Beef Extract	1.0	g
Peptone	10.0	g
D-Mannitol	10.0	g
Sodium Chloride	10.0	g
Phenol Red	25.0	mg
Agar	15.0	g
Egg Yolk Enrichment 50%		
Polymyxin B		

Procedure

- Antimicrobic Vial P (Polymyxin B) Preparation

Polymyxin B powder was rehydrated by aseptically adding 5 ml of sterile distilled water. Rotate in an end-over-end motion to dissolve Polymyxin B powder completely.

All of ingredients excepted agar, Polymyxin B and Egg Yolk Enrichment were dissolved in 900 ml of distilled water. Mix it thoroughly and adjust pH to 7.2 ± 0.1. 225 ml of MYP medium and 1.5% of agar were added to 500 ml flasks. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes. Cool to 45 – 50°C and aseptically add 12.5 ml of Egg Yolk Enrichment 50% and 4.1 ml of Antimicrobic Vial P (25,000 units of polymyxin B). Mix thoroughly.

14. PALCAM Medium Base

Bacto Columbia Blood Agar Base	39.0	g
Bacto Mannitol	10.0	g
Bacto Dextrose	0.5	g
Esculin	1.0	g
Ferric Ammonium Citrate	0.5	g
Lithium Chloride	15.0	g
Phenol Red	0.08	g
Acriflavin HCl	5.0	mg
Polymyxin B Sulfate	0.01	g
Agar	20.0	g
PALCAM Antimicrobic Supplement		

Procedure

- PALCAM Antimicrobic Supplement Preparation

Aseptically add 10 ml sterile purified water to the vial and shake to dissolve the PALCAM Antimicrobic Supplement powder completely.

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.2±0.2. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes. Cool to 45 - 50°C and aseptically add 2 ml PALCAM Antimicrobic Supplement. Mix it thoroughly.

15. Phenol Red Broth Base

Beef extract	5.0	g
Proteose peptone No.3	10.0	g
Sodium Chloride	5.0	g
Phenol red	0.018	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. 1% of carbohydrate sources were added. Mix it thoroughly. Dispense into tubes and insert Durham tubes when gas fermentation is to be recorded. Adjust pH to 6.8±0.2. Autoclave at 110°C for 15 minutes.

16. Simmons Citrate Agar

Ammonium Dihydrogen Phosphate	1.0	g
Dipotassium Phosphate	1.0	g
Sodium Chloride	5.0	g
Sodium Citrate	2.0	g
Magnesium Sulfate	0.2	g
Agar	15.0	g
Bromthymol Blue	0.08	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 6.9±0.2. Heat in order to completely dissolve. Dispense into tubes and autoclave at 121°C for 15 minutes. Cool in a slanted position.

17. Tetrathionate Broth Base

Proteose Peptone	2.5	g
Pancreatic Digest of Casein	2.5	g
Oxgall	1.0	g
Sodium Thiosulfate	30.0	g
Calcium Carbonate	10.0	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 8.4±0.2. Heat to boil. Cool to below 60°C and add 2 ml of iodine solution (6.0 g of iodine crystals and 5.0 g of potassium iodide in 20.0 ml of water). Do not reheat medium after adding iodine and autoclave.

18. 0.75 % TSAYE

Procedure

Consist of the same ingredients of Tryptic Soy Broth. Both of yeast extract and agar were added 0.75 g in 100 ml of Tryptic Soy Broth. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes

19. Triple Sugar Iron (TSI) Agar

Beef Extract	3.0	g
Yeast Extract	3.0	g
Pancreatic Digest of Casein	15.0	g
Proteose Peptone No.3	5.0	g
Dextrose	1.0	g
Lactose	10.0	g
Sucrose	10.0	g
Ferrous Sulfate	0.2	g
Sodium Chloride	5.0	g
Sodium Thiosulfate	0.3	g
Agar	12.0	g
Phenol Red	24.0	mg

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.4±0.2. Heat for 1 minute to completely dissolve. Dispense into tubes and autoclave at 121°C for 15 minutes. Cool in a slanted position so that deep butts are formed.

20. Tryptic Soy Agar (TSA)

Procedure

Consist of the same ingredients of Tryptic Soy Broth. 16 g of agar was added in 1 L of Tryptic Soy Broth. Heat in order to completely dissolve and autoclave at 121°C for 15 minutes

21. Tryptic Soy Broth (TSB)

Pancreatic Digest of Casein	17.0	g
Enzymatic Digest of Soybean Meal	3.0	g
Sodium Chloride	5.0	g
Dipotassium Phosphate	2.5	g
Dextrose	2.5	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and autoclave at 121°C for 15 minutes.

22. UVM Modified Listeria Enrichment Broth

Pancreatic Digest of Casein	5.0	g
Proteose Peptone No. 3	5.0	g
Beef Extract	5.0	g
Yeast Extract	5.0	g
Sodium Chloride	20.0	g
Disodium Phosphate	9.6	g
Monopotassium Phosphate	1.35	g
Esculin	1.0	g
Nalidixic Acid	0.02	g
Acriflavine HCl	12.0	mg

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.2±0.2. Autoclave at 121°C for 15 minutes.

23. Xylose-Lysine Deoxycholate (X.L.D) Agar

Yeast extract	3.0	g
L-Lysine HCl	5.0	g
Xylose	3.75	g
Lactose	7.5	g
Sucrose	7.5	g
Sodium desoxycholate	1.0	g
Sodium chloride	5.0	g
Sodium thiosulphate	6.8	g
Ferric ammonium citrate	0.8	g
Phenol red	0.08	g
Agar	12.5	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust pH to 7.4 $\pm$ 0.2. Heat with frequent agitation and boil to completely dissolve the powder (Do not overheat and autoclave).

Appendix B

Chemical and Reagents Preparation

1. Buffered Peptone Water

Peptone	10.0	g
Sodium Chloride (NaCl)	5.0	g
Disodium Phosphate (Na_2HPO_4)	3.5	g
Monopotassium Phosphate (KH_2PO_4)	1.5	g

Procedure

All of ingredients were dissolved in 1 L of distilled water. Mix it thoroughly and adjust to pH 7.2 $\pm$ 0.2. Autoclave at 121°C for 15 minutes.

2. Crystal violet solution

Solution A

Crystal violet	3.0	g
95% Ethyl alcohol	20.0	ml

Solution B

Ammonium oxalate	0.8	g
Distilled water	50.0	ml

Procedure

Dissolve 3 g crystal violet in 20 ml 95 % ethyl alcohol (solution A) and mix with 50 ml of ammonium oxalate solution (solution B). Filtrate before use.

3. Gram's iodine solution

Iodine crystal	1.0	g
Potassium iodide (KI)	2.0	g
Distilled water	300.0	ml

Procedure

Dissolve 2 g of potassium iodide to 25 ml distilled water and mix it thoroughly. Add 1 g of iodine to the potassium iodide solution and mix well. Then, add 275 ml distilled water. Keep the solution in dark bottle.

4. 3% Hydrogenperoxide

30% Hydrogen peroxide (H_2O_2)	3.0	ml
Distilled water	27.0	ml

Procedure

3 ml of 30% hydrogen peroxide was added in distilled water. Mix it thoroughly. Keep the solution in grey bottle at 4 °C.

5. 1 N hydrochloric acid

Hydrochloric acid (conc) (HCl)	8.29	mL
Distilled water	91.71	mL

Procedure

Mix hydrochloric acid and distilled water to make 1 N HCl

6. Kovac's reagent

Paradimethylaminobenzaldehyde	3.0	g
Butanol	75.0	ml
Hydrochloric acid (conc)	25.0	ml

Procedure

Dissolve paradimethylaminobenzaldehyde in butanol at 50-55 °C. Cool to room temperature and add hydrochloric acid. Mix thoroughly and keep the solution in dark bottle.

7. Methyl red solution

Methyl Red	0.02	g
95% Ethyl Alcohol	60.0	ml
Sterile Deionized Water	40.0	ml

Procedure

Dissolve methyl red in 95% ethyl alcohol and add sterile deionized water for the final volume 100 ml. Keep the solution in dark bottle

8. Phosphate Buffered solution pH 7.0

Sodiumdihydrogenphosphate (NaH_2PO_4)	87.36	g
Disodiumhydrogenphosphate (Na_2HPO_4)	46.14	g
Distilled water	1	L

Procedure

All of ingredients were dissolved in distilled water. Mix it thoroughly and adjust to pH 7.0. Distilled water was added to reach 1 L final volume. Autoclave at 121°C for 15 minutes.

9. Safranin O stock solution

Safranin	2.5	g
95% Ethyl alcohol	100.0	ml

Procedure

Dissolve 2.5 g safranin O in 100 ml of 95% ethyl alcohol. For use, 10 ml of solution should be diluted with 90 ml of distilled water. Filtrate before use.

10. 0.85% w/v sodium chloride

Sodium Chloride (NaCl)	8.5	g
Distilled water	1	L

Procedure

Sodium chloride was dissolved in distilled water. Distilled water was added to reach 1 L final volume. Autoclave at 121°C for 15 minutes.

11. 1 N Sodium hydroxide

Sodium hydroxide (NaOH)	40.0	g
Distilled water	1	L

Procedure

Sodium hydroxide was dissolved in distilled water. Distilled water was added to reach 1 L final volume.

12. Tryptone Water

Tryptone	10.0	g
Sodium Chloride (NaCl)	5.0	g

Procedure

Tryptone and Sodium Chloride were dissolved in 1 L of distilled water. Mix it thoroughly. Autoclave at 121°C for 15 minutes.

13. Voges-Proskauer (VP) test solution

Solution A

α-napthol	5.0	ml
95% Ethyl Alcohol	100.0	ml

Procedure

Dissolve alpha-napthol in 95% ethyl Alcohol. Mix thoroughly and keep the solution in dark bottle.

Solution B

Potassium hydroxide	40.0	g
Distilled water	100.0	ml

Procedure

Dissolve potassium hydroxide in distilled water. Mix thoroughly and keep the solution in dark bottle.

Appendix C

Identification of Lactic Acid Bacteria

1. Gram staining

Add a drop or a few loopful of distilled water on the slide and aseptically transfer a minute amount of single colony to distilled water. Smear a very thin layer onto the slide and fix it with a gentle flame. Add crystal violet (appendix B) over the fixed culture. Let stand for 1 minute. Pour off the stain and gently rinse the excess stain with distilled water. Add the iodine solution (appendix B) on the smear. Let stand for 1 minute. Pour off the iodine solution and rinse the slide with distilled water. Add a few drops of 95% ethanol for decolourization 10-20 seconds and rinse it off with water. Then, stain with safranin O (appendix B). Let stand for 1 minute. Wash off the solution with distilled water. The excess water was removed by tissue paper. Examine the morphology and Gram stain of bacteria under the microscope.

2. Catalase test

Transfer a single colony of the isolate and smear onto the slide. Add a few drops of 3% H_2O_2 (appendix B) cover a smear and observe the gas bubbles formation. Appearance of gas bubbles indicates a positive test by comparison with *Bacillus cereus* ATCC 1729 were obtained from Department of Microbiology, Faculty of Science, Chulalongkorn University as the positive control. No appearance of gas bubbles indicates a negative test.

3. Gas production test

Single colony of the isolate was cultured in Phenol Red Base broth with 1% of glucose (appendix A) inserted with durham tube. Incubate at 37°C, 24-48 hours. Homofermentative bacteria could not produce carbon dioxide gas (CO_2), so gas

bubbles were not observed in durham tube. In contrast, heterofermentative bacteria could produce carbon dioxide gas (CO_2), so gas bubbles were observed in durham tube.

4. Motility test

Single colony of the isolate was inoculated by stabbing through center of the motility medium (appendix A) with needle to the depth of the medium and incubated at 37°C under anaerobic for 18-48 hours. Motility was observed visually by diffuse growth spreading from the line of inoculation, while non-motile organisms grew only along the line of inoculation.

5. Growth of Lactic acid Bacteria in different of sodium chloride concentration

LAB isolate was cultured in MRS broth supplemented with bromocresol purple used as the indicator. Concentration of sodium chloride was prepared for this experiment at 4% and 6.5% (w/v). pH of MRS broth was adjusted to 6.5-6.8. Incubate at 37°C for 5 days. Growth of the isolate was observed from the color change in the medium from purple to yellow.

6. Growth of Lactic Acid Bacteria in different of temperature

LAB isolate was cultured in MRS broth supplemented with bromocresol purple used as the indicator. pH of MRS broth was adjusted to 6.5-6.8. Incubate at 10°C and 45°C for 5 days. Growth of the isolate was observed from the color change in the medium from purple to yellow.

7. Growth of Lactic Acid Bacteria in different of pH values

LAB isolate was cultured in MRS broth preparing in different pH value including pH 4.4 and 9.6 and incubate at 37°C for 5 days. Growth of the isolate was observed from the turbidity of medium.

8. Carbohydrate fermentation by API 50CHL

Carbohydrate fermentation was examined by using API 50 CH (BioMérieux, France) and API 50 CHL Medium (BioMérieux, France). API 50 CH strip consisted of 50 microtubes used to study the fermentation of 49 carbohydrates. Composition of API 50 CH strip was given in the Table C1 API 50 CH was used in conjunction with API 50 CHL Medium for the identification of *Lactobacillus* and related genera.

Table C1 49 carbohydrates composition of API 50 CH strip

Tube	Test	Active ingredients	QTY (mg/cup.)
0		CONTROL	1.64
1	GLY	GLYcerol	1.44
2	ERY	ERYthritol	1.4
3	DARA	D-ARAbinose	1.4
4	LARA	L-ARAbinose	1.4
5	RIB	D-RIBose	1.4
6	DXYL	D-XYLose	1.4
7	LXYL	L-XYLose	1.4
8	ADO	D-ADOnitol	1.36
9	MDX	Methyl-ßD-Xylopyranoside	1.28
10	GAL	D-GALactose	1.4
11	GLU	D-GLUcose	1.56

Tube	Test	Active ingredients	QTY (mg/cup.)
12	FRU	D-FRUctose	1.4
13	MNE	D-MaNnosE	1.4
14	SBE	L-SorBosE	1.4
15	RHA	L-RHAmnose	1.36
16	DUL	DULcitol	1.36
17	INO	INOsitol	1.4
18	MAN	D-MANnitol	1.36
19	SOR	D-SORbitol	1.36
20	MDM	Methyl-αD-Mannopyranoside	1.28
21	MDG	Methyl-αD-Glucopyranoside	1.28
22	NAG	N-AcetylGlucosamine	1.28
23	AMY	AMYgdalin	1.08
24	ARB	ARButin	1.08
25	ESC	ESCulin ferric citrate	1.16 0.152
26	SAL	SALicin	1.04
27	CEL	D-CELlobiose	1.32
28	MAL	D-MALtose	1.4
29	LAC	D-LACtose (bovine origin)	1.4
30	MEL	D-MELibiose	1.32
31	SAC	D-SACcharose (sucrose)	1.32
32	TRE	D-TREhalose	1.32
33	INU	INUlin	1.28
34	MLZ	D-MeLeZitose	1.32
35	RAF	D-RAFfinose	1.56

Tube	Test	Active ingredients	QTY (mg/cup.)
36	AMD	AmiDon (starch)	1.28
37	GLYG	GLYcoGen	1.28
38	XLT	XyLiTol	1.4
39	GEN	GENtiobiose	0.5
40	TUR	D-TURanose	1.32
41	LYX	D-LYXose	1.4
42	TAG	D-TAGatose	1.4
43	DFUC	D-FUCose	1.28
44	LFUC	L-FUCose	1.28
45	DARL	D-ARabitoL	1.4
46	LARL	L-ARabitoL	1.4
47	GNT	potassium GlucoNaTe	1.84
48	2KG	potassium 2-KetoGluconate	2.12
49	5KG	potassium 5-KetoGluconate	1.8

8.1 Selection of the colonies

The selected isolate was cultured on MRS agar medium and incubate anaerobically at 37°C for 24 hours under anaerobic condition. If freeze dried stock cultures are used, subculture twice in MRS broth before isolation on MRS agar medium. Some characteristic of lactic acid bacteria were checked such as Gram staining, catalase test, spore-formation, the condition of growth and morphology under microscope. Lactic acid bacteria are Gram positive bacteria. The morphology is rod or cocci. They can not produce catalase enzyme and non-spore forming bacteria. They grow on MRS agar at anaerobic, facultative or occasionally obligate condition.

8.2 Preparation of the strip

Each strip is made up of 5 smaller strips each containing 10 numbered tubes (0-9, 10-19, 20-29, 30-39 and 40-49). Incubation box (tray and lid) was prepared and recorded the reference of the strain on the elongated flap of the tray. 10 ml of distilled water or demineralized water was distributed into the honeycombed wells of the tray to create a humid atmosphere. 4 smaller strips (0-9, 10-19, 20-29 and 30-39) were placed in the incubation tray, and then the last smaller strip (40-49) was placed next to the others in the incubation tray to complete the strip.

8.3 Preparation of the inoculum

Test tube containing 2 ml (tube A) and 5 ml of sterile distilled water (tube B) was prepared. All the bacteria of the culture on MRS agar were picked up by using a swab and were made a heavy suspension (S) in tube A. Then, tube B was adjusted a turbidity equivalent to 2 McFarland by transferring the suspension from tube A. A certain number of drops were recorded (n). API 50 CHL Medium was inoculated by adding twice the number of drops of suspension S (i.e. 2n) and homogenized.

8.4 Inoculation of the strip

Fill the tubes (not the cupules) with the inoculated API 50 CHL Medium, and avoid the formation of bubbles. Overlay all of the tests with mineral oil and incubate aerobically at 30 or 37°C, for 48 hours.

8.5 Reading the strip

The result of carbohydrate fermentation was read after 24 and 48 hours of incubation. A positive test corresponds to acidic condition that the bromocresol purple indicator contained in the medium was changed to yellow. If the medium was changed from purple to yellow, positive result was recorded. In contrast, negative result was

recorded when the color of medium was not changed. For the positive result of Esculin test (tube no. 25), a change from purple to black was observed. All of results were recorded on the result sheets.

8.6 Interpretation

The result of biochemical profile obtained for the strain can be identified by using identification software in http://apiweb.biomerieux.com.

Appendix D

Biochemical test

IMViC test

The IMViC tests are a group of biochemical tests used for identifying microorganism in the coliform group such as *Escherichia coli* and *Enterobacter* sp. The IMViC tests include indole production, Methyl red test, Voges-Proskauer test and Citrate utilization test.

1. Indole production test

Single colony of the unknown isolate from EMB agar plate (appendix A) was cultured in 1% tryptone broth. (appendix A). Incubate at 37°C for 18-24 hours. Add 5 drops of Kovacs' reagent and shake gently. Formation of a red ring over the broth indicates indole presence.

2. Methyl red and Voges-Proskauer (MR-VP test)

Single colony of the unknown isolate from EMB agar plate was cultured in MR-VP broth 1 ml (appendix A). Incubate at 37°C for 18-24 hours. Dispense MR-VP broth into 2 tubes.

For Methyl red test : add 5 drops of methyl red solution (appendix B) to MR-VP broth. Positive and negative results give red and yellow color of the medium, respectively.

For Voges-Proskauer test : add 5 drops of α-naphthol (appendix B) and 2 drops of 16%KOH (Appendix B) to MR-VP broth. Shake well after the addition of each reagent. Interpret the color result within 1 hour of adding the reagents. Positive and negative results give red and yellow color of the medium, respectively.

3. Citrate utilization test

Single colony of the unknown isolate from EMB agar plate was streaked on the surface of Simmons Citrate agar slant (appendix A). Incubate at 37°C for 18-24 hours. A positive result gives blue color in the slant and a negative result gives no change in color.

4. Triple Sugar Iron (TSI) test

Single colony from Hektoen Enteric agar was stab into the TSI medium (appendix A) in the butt of the tube, and then streak back on the surface of the slant. Incubate at 37°C for 18-24 hours under aerobic condition. Do not interpret the color result longer than 24 hours. TSI tests include carbohydrate fermentation, Hydrogen sulfide production and Gas production

A yellow (acidic) color in the slant and butt indicates that the microorganism ferments dextrose, lactose and/or sucrose.

A red (alkaline) color in the slant and butt indicates that the microorganism is a nonfermenter.

Hydrogen sulfide production results in a black precipitate in the butt of the tube.

Gas production is indicated by splitting and cracking of the medium.

5. Lysine Iron Agar (LIA)

Single colony from Hektoen Enteric agar was stab into the LIA medium (appendix A) in the butt of the tube, and then streak back on the surface of the slant. Incubate at 37°C for 18-48 hours under aerobic condition.

Lysine decarboxylation is detected in the butt by an alkaline (purple) reaction. A negative reaction is yellow butt that indicates fermentation of dextrose only.

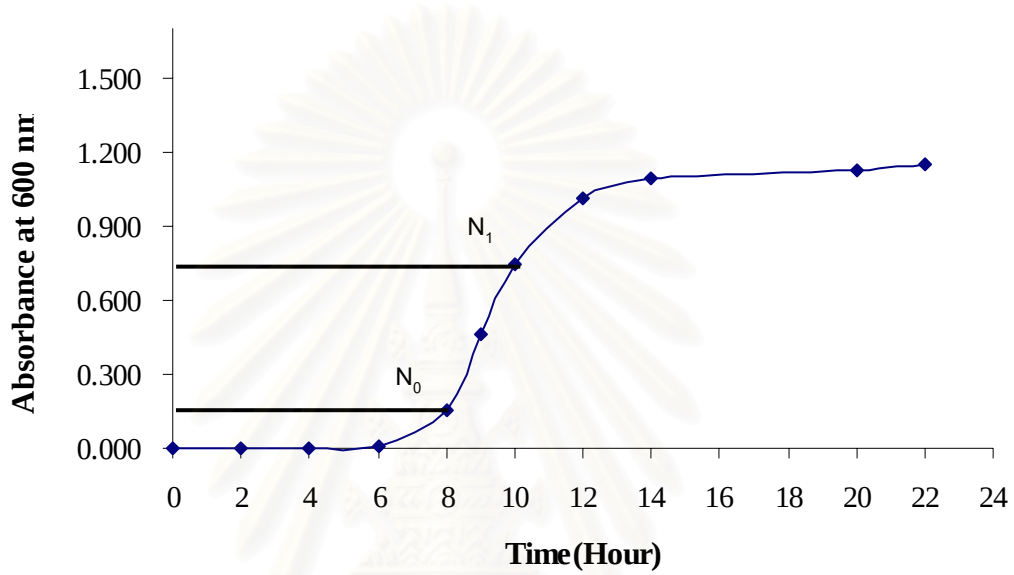
Lysine deamination is detected by a red slant. A negative reaction is purple slant.

Hydrogen sulfide production is detected by the formation of a black precipitate. Hydrogen sulfide may not be detected in this medium by organisms that are negative for lysine decarboxylase activity.

Appendix E

Calculation

1. Calculation for specific growth rate



Calculation

$$\mu = \frac{(\ln N_1 - \ln N_0)}{(t_1 - t_0)}$$

When

N_1 = Absorbance at time 1

N_0 = Absorbance at time 0

T_1 = Time 1

T_0 = Time 0

2. Calculation of doubling time

$$T = \frac{0.693}{\mu}$$

When

T = Doubling time (hour)

μ = Specific growth rate ($hour^{-1}$)

3. Calculation of bacteriocin activity

Bacteriocin activity ($AUml^{-1}$) = Reciprocal of the highest dilution showing inhibition of the indicator strain x 100

4. Calculation of total acidity (%w/v)

$$\text{Percentage of Total acidity} = \frac{(V) \times (N) \times (MW) \times 100\%}{1000 \times (U)}$$

When

V = Quantity of standard NaOH for titration (ml)

N = Concentration of standard NaOH (Molarity)

U = Quantity of sample for titration (g)

MW = Molecular weight of lactic acid

In this study, total acid of nham inoculated with *P. pentosaceus* as starter culture was assumed as lactic acid and shown in term of relative of total acid increase, because *P. pentosaceus* is a homofermentative-LAB. In fact, fermentation of nham occurred by mixed-cultures from raw materials, not only lactic acid but also many kinds

of acid were produced. For correct calculation of lactic acid and mixed acids concentration, high performance liquid chromatography (HPLC) should be the alternative way for measuring acid production during fermentation.

Appendix F

Data of experiments

1. Table F1 LAB isolated from twenty samples of nham collected from Suddhiluck Innofood Co.,Ltd

Sample number	Isolate number	Catalase test	Gram staining	Motility	Cell forms	Cell arrangement
1	NSL3-1	-	+	-	Cocci	pairs
	NSL12-1	-	+	-	Cocci	single
	NSL14-1	-	+	-	Rods	clusters
	NSL15-1	-	+	-	Cocci or Oval	single or pairs
	NSL16-1	-	+	-	Cocci	single or pairs
	NSL18-1	-	+	-	Cocci	pairs or clusters
	NSL19-1	-	+	-	Rods	single
	NSL20-1	-	+	-	Cocci	short chains
	NSL21-1	-	+	-	Cocci	pairs
2	NSL6-1	-	+	-	Cocci	chains
	NSL7-1	-	+	-	Cocci	pairs, short chains
	NSL8-1	-	+	-	Rods	pairs, clusters
	NSL9-1	-	+	-	Cocci	single
	NSL22-1	-	+	-	Rods	chains
	NSL25-1	-	+	-	Cocci or Oval	chains
	NSL26-1	-	+	-	Rods	clusters
3	NSL30-1	-	+	-	Rods	pairs, clusters
	NSL31-1	-	+	-	Rods	chains
	NSL32-1	-	+	-	Rods	single, clusters

Sample number	Isolate number	Catalase test	Gram staining	Motility	Cell forms	Cell arrangement
	NSL33-1	-	+	-	Rods	single, pairs
	NSL36-1	-	+	-	Rods	chains
	NSL37-1	-	+	-	Rods	single, clusters
	NSL39-1	-	+	-	Cocci or Oval	chains
	NSL45-1	-	+	-	Rods	pairs, clusters
4	NSL11-1	-	+	-	Rods	short chains
	NSL41-1	-	+	-	Rods	pairs, clusters
5	NSL1-2	-	+	-	Cocci	clusters
	NSL3-2	-	+	-	Rods	pairs, short chains
	NSL4-2	-	+	-	Cocci	clusters
	NSL5-2	-	+	-	Cocci or Oval	single, pairs
	NSL6-2	-	+	-	Rods	pairs, clusters
	NSL17-2	-	+	-	Cocci	clusters
	NSL18-2	-	+	-	Cocci	clusters
	NSL19-2	-	+	-	Cocci	pairs, tetrads
	NSL25-2	-	+	-	Cocci	clusters
6	NSL7-2	-	+	-	Cocci	pairs, short chains
	NSL8-2	-	+	-	Cocci	pairs
	NSL9-2	-	+	-	Rods	single, pairs
	NSL10-2	-	+	-	Rods	short chains
	NSL12-2	-	+	-	Cocci	pairs, clusters
	NSL20-2	-	+	-	Rods	clusters
7	NSL13-2	-	+	-	Cocci	pairs, tetrads
	NSL14-2	-	+	-	Cocci	chains
	NSL15-2	-	+	-	Rods	clusters

Sample number	Isolate number	Catalase test	Gram staining	Motility	Cell forms	Cell arrangement
	NSL21-2	-	+	-	Cocci	pairs, short chains
	NSL24-2	-	+	-	Cocci	pairs, clusters
8	NSL23-2	-	+	-	Rods	chains
	NSL15-2	-	+	-	Cocci	clusters
9	NSL3-3	-	+	-	Cocci	pairs, short chains
	NSL4-3	-	+	-	Cocci	pairs, clusters
	NSL15-3	-	+	-	Cocci	single, pairs
	NSL24-3	-	+	-	Cocci	pairs, short chains
	NSL25-3	-	+	-	Cocci	pairs, clusters
10	NSL1-3	-	+	-	Rods	pairs, clusters
	NSL5-3	-	+	-	Cocci	single, pairs
	NSL6-3	-	+	-	Rods	single, pairs
	NSL26-3	-	+	-	Cocci	pairs, clusters
	NSL27-3	-	+	-	Rods	single, pairs
11	NSL7-3	-	+	-	Cocci	pairs, short chains
	NSL8-3	-	+	-	Rods	pairs, clusters
	NSL18-3	-	+	-	Rods	pairs, clusters
	NSL28-3	-	+	-	Cocci	pairs, tetrads
	NSL29-3	-	+	-	Cocci	pairs, clusters
12	NSL2-3	-	+	-	Rods	pairs, clusters
	NSL9-3	-	+	-	Rods	single, pairs
	NSL10-3	-	+	-	Cocci	pairs, clusters
	NSL20-3	-	+	-	Rods	pairs, clusters
	NSL21-3	-	+	-	Cocci	pairs, clusters
	NSL30-3	-	+	-	Cocci	pairs, clusters

Sample number	Isolate number	Catalase test	Gram staining	Motility	Cell forms	Cell arrangement
	NSL31-3	-	+	-	Cocci	pairs, tetrads
13	NSL11-3	-	+	-	Rods	single, pairs
	NSL22-3	-	+	-	Rods	clusters
	NSL32-3	-	+	-	Rods	pairs, short chains
	NSL33-3	-	+	-	Cocci	pairs
14	NSL13-3	-	+	-	Cocci	clusters
	NSL23-3	-	+	-	Cocci	pairs, clusters
	NSL34-3	-	+	-	Rods	pairs, short chains
	NSL35-3	-	+	-	Cocci	pairs, tetrads
15	NSL1-4	-	+	-	Coccobacilli	pairs, clusters
	NSL2-4	-	+	-	Cocci	pairs, tetrads
	NSL11-4	-	+	-	Rods	single, pairs
	NSL12-4	-	+	-	Rods	single, pairs
	NSL23-4	-	+	-	Cocci	single, pairs
	NSL30-4	-	+	-	Rods	single, clusters
	NSL31-4	-	+	-	Rods	single, pairs
16	NSL4-4	-	+	-	Cocci	pairs, clusters
	NSL13-4	-	+	-	Cocci	pairs, clusters
	NSL14-4	-	+	-	Cocci	pairs, clusters
	NSL15-4	-	+	-	Rods	single, pairs
	NSL25-4	-	+	-	Cocci	pairs, tetrads
17	NSL5-4	-	+	-	Cocci	pairs, short chains
	NSL6-4	-	+	-	Cocci	pairs, short chains
	NSL16-4	-	+	-	Cocci	pairs, short chains
	NSL17-4	-	+	-	Rods	single, clusters

Sample number	Isolate number	Catalase test	Gram staining	Motility	Cell forms	Cell arrangement
	NSL26-4	-	+	-	Cocci	single, clusters
18	NSL7-4	-	+	-	Cocci	pairs, short chains
	NSL18-4	-	+	-	Cocci	pairs, tetrads
	NSL19-4	-	+	-	Cocci	pairs, clusters
	NSL27-4	-	+	-	Cocci	pairs, clusters
19	NSL8-4	-	+	-	Cocci	pairs, chains
	NSL9-4	-	+	-	Cocci	pairs, short chains
	NSL20-4	-	+	-	Cocci	pairs, chains
	NSL24-4	-	+	-	Rods	single, pairs
	NSL28-4	-	+	-	Cocci	single, pairs
20	NSL10-4	-	+	-	Cocci	pairs, clusters
	NSL21-4	-	+	-	Cocci	pairs, chains
	NSL22-4	-	+	-	Rods	single, pairs
	NSL29-4	-	+	-	Cocci	pairs, tetrads

Catalase test - = not produce catalase; Gram staining + = Gram-positive; Motility - = non-motile

2. Table F2 Bacteriocin-producing LAB against indicator strains isolated by spotted test method

Isolate number	Indicator strains				
	S.aureus	*B.cereus*	*L.monocytogenes*	*E.coli*	*S.*Typhimurium
NSL8-1	+	-	-	-	-
NSL11-1	+	-	-	-	-
NSL12-1	+	-	-	-	-
NSL14-1	+	-	-	-	-
NSL15-1	+	-	-	-	-

Isolate number	Indicator strains				
	S.aureus	*B.cereus*	*L.monocytogenes*	*E.coli*	*S.*Typhimurium
NSL16-1	+	-	-	-	-
NSL18-1	+	-	+	-	-
NSL20-1	+	-	+	-	-
NSL21-1	+	-	+	-	-
NSL22-1	+	+	-	-	-
NSL26-1	+	-	-	-	-
NSL31-1	+	-	-	-	-
NSL33-1	+	-	-	-	+
NSL37-1	+	-	-	-	-
NSL39-1	+	-	-	-	-
NSL41-1	+	-	-	-	-
NSL45-1	+	-	-	-	-
NSL1-2	+	+	-	-	-
NSL4-2	+	-	-	-	-
NSL5-2	+	+	-	-	-
NSL6-2	+	-	-	+	-
NSL7-2	+	-	-	-	-
NSL8-2	+	-	-	-	-
NSL9-2	+	-	-	-	-
NSL10-2	+	-	-	-	-
NSL12-2	+	-	-	-	-
NSL13-2	+	-	+	-	-
NSL14-2	+	-	-	-	-
NSL15-2	+	-	-	-	-
NSL17-2	+	-	-	-	-
NSL18-2	+	-	-	-	-

Isolate number	Indicator strains				
	S.aureus	*B.cereus*	*L.monocytogenes*	*E.coli*	*S.*Typhimurium
NSL19-2	+	-	+	-	-
NSL20-2	+	-	-	-	-
NSL21-2	+	-	-	-	-
NSL23-2	+	-	-	-	-
NSL24-2	+	-	-	-	-
NSL25-2	+	-	-	+	-
NSL9-3	+	-	-	-	-
NSL13-3	+	-	-	-	-
NSL15-3	+	+	-	-	-
NSL20-3	+	-	-	-	-
NSL23-3	+	-	-	-	-
NSL25-3	+	-	-	-	-
NSL26-3	+	-	-	-	-
NSL27-3	+	-	-	-	-
NSL29-3	+	-	-	-	-
NSL31-3	+	-	-	-	-
NSL33-3	+	-	-	-	-
NSL34-3	+	-	-	-	-
NSL1-4	+	-	-	+	-
NSL2-4	+	-	-	-	-
NSL5-4	+	-	-	-	-
NSL6-4	+	-	-	-	-
NSL8-4	+	-	-	-	-
NSL9-4	+	-	-	-	-
NSL13-4	+	-	-	-	-
NSL14-4	+	+	-	-	-

Isolate number	Indicator strains				
	S.aureus	*B.cereus*	*L.monocytogenes*	*E.coli*	*S.*Typhimurium
NSL15-4	+	-	-	-	-
NSL16-4	+	-	-	-	-
NSL19-4	+	-	-	-	-
NSL20-4	+	-	-	-	-
NSL25-4	+	-	-	-	-
NSL26-4	+	-	-	-	-
NSL30-4	+	+	-	-	-
NSL31-4	+	-	-	-	-

+ = positive; - = negative

3. Table F3 Growth of *Staphylococcus aureus* ATCC 25923 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.003 ± 0.002	4.46
2	0.004 ± 0.001	5.54
4	0.015 ± 0.003	6.70
5	0.103 ± 0.006	7.44
6	0.360 ± 0.008	8.02
6.5	0.481 ± 0.016	ND
7	0.691 ± 0.023	8.68
7.5	0.956 ± 0.025	ND
8	1.202 ± 0.019	9.16
8.5	1.401 ± 0.009	ND
9	1.496 ± 0.010	9.42
9.5	1.568 ± 0.014	ND
10	1.620 ± 0.011	9.32

ND means “Not Done”

The values shown in the table were averaged from triplicate

4. Table F4 Growth of *Listeria monocytogenes* DMST 17303 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.004±0.000	6.11
1	0.003±0.002	ND
2	0.006±0.004	ND
3	0.019±0.003	ND
4	0.057±0.005	8.10
5	0.172±0.015	8.41
5.5	0.270±0.014	ND
6	0.435±0.015	8.91
6.5	0.591±0.010	9.19
7	0.687±0.005	9.35
7.5	0.741±0.002	9.41
8	0.782±0.002	9.38
8.5	0.812±0.002	ND
9	0.843±0.003	9.44
9.5	0.871±0.006	ND
10	0.893±0.006	9.45

ND means “Not Done”

The values shown in the table were averaged from triplicate

5. Table F5 Growth of *Bacillus cereus* ATCC 1729 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.004 ± 0.000	3.30
1	0.004 ± 0.000	4.36
2	0.005 ± 0.001	5.72
3	0.044 ± 0.002	6.93
3.5	0.172 ± 0.015	ND
4	0.587 ± 0.042	8.00
4.5	1.010 ± 0.043	ND
5	1.192 ± 0.034	8.51
5.5	1.318 ± 0.023	ND
6	1.358 ± 0.031	8.89
6.5	1.424 ± 0.030	ND
7	1.432 ± 0.028	8.91

ND means “Not Done”

The values shown in the table were averaged from triplicate

6. Table F6 Growth of *Escherichia coli* ATCC 25922 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.000 ± 0.000	3.99
2	0.000 ± 0.000	5.12
4	0.007 ± 0.003	6.68
5	0.044 ± 0.002	8.06
5.5	0.138 ± 0.029	ND
6	0.289 ± 0.054	8.45
6.5	0.497 ± 0.037	ND
7	0.777 ± 0.072	8.98
7.5	1.006 ± 0.059	ND
8	1.135 ± 0.010	9.34
8.5	1.253 ± 0.058	ND
9	1.323 ± 0.051	9.76
9.5	1.367 ± 0.060	ND
10	1.412 ± 0.065	9.68
10.5	1.469 ± 0.090	ND
11	1.528 ± 0.084	ND

ND means “Not Done”

The values shown in the table were averaged from triplicate

7. Table F7 Growth of *Salmonella* Typhimurium ATCC 13311 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.000 ± 0.000	4.37
2	0.000 ± 0.000	5.44
4	0.014 ± 0.005	ND
5	0.096 ± 0.021	8.00
5.5	0.167 ± 0.018	ND
6	0.378 ± 0.017	7.81
6.5	0.552 ± 0.027	ND
7	0.802 ± 0.045	8.77
7.5	1.057 ± 0.025	ND
8	1.205 ± 0.008	9.57
8.5	1.279 ± 0.016	ND
9	1.356 ± 0.021	9.81
9.5	1.430 ± 0.018	ND
10	1.489 ± 0.024	10.18
10.5	1.540 ± 0.040	ND
11	1.608 ± 0.036	ND

ND means “Not Done”

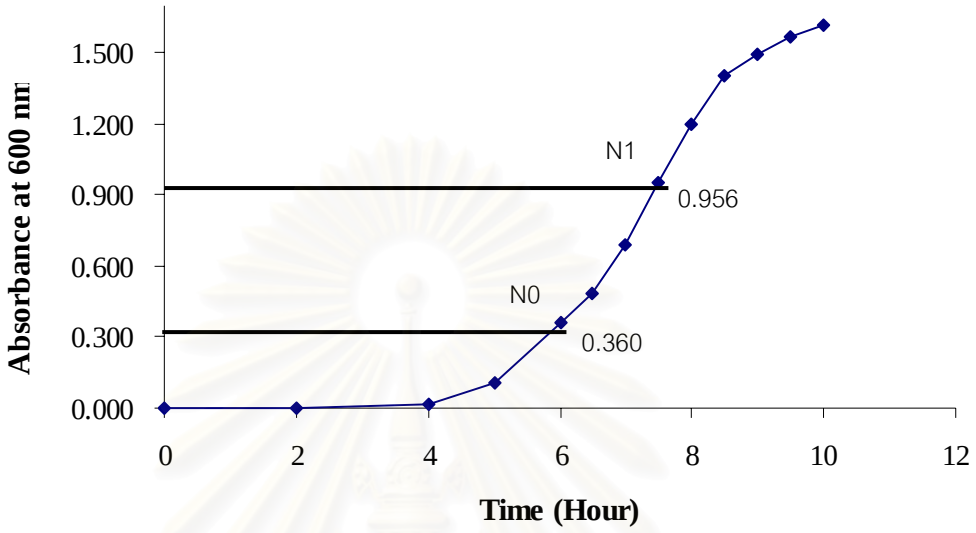
The values shown in the table were averaged from triplicate

8. Table F8 Growth of *Pediococcus pentosaceus* NSL13-2 determined by following turbidity of absorbance at 600 nm and bacteria count at different time

Time (Hour)	Absorbance (600 nm)	Total bacteria count (log $CFUml^{-1}$)
0	0.000 ± 0.001	3.84
2	0.001 ± 0.001	4.44
4	0.002 ± 0.003	6.47
6	0.017 ± 0.006	6.64
7	0.052 ± 0.007	7.02
8	0.165 ± 0.012	8.31
9	0.446 ± 0.024	8.89
10	0.982 ± 0.034	9.26
11	1.373 ± 0.040	9.45
12	1.539 ± 0.042	9.58
14	1.749 ± 0.049	9.57
18	1.803 ± 0.054	9.47
20	1.766 ± 0.043	9.73

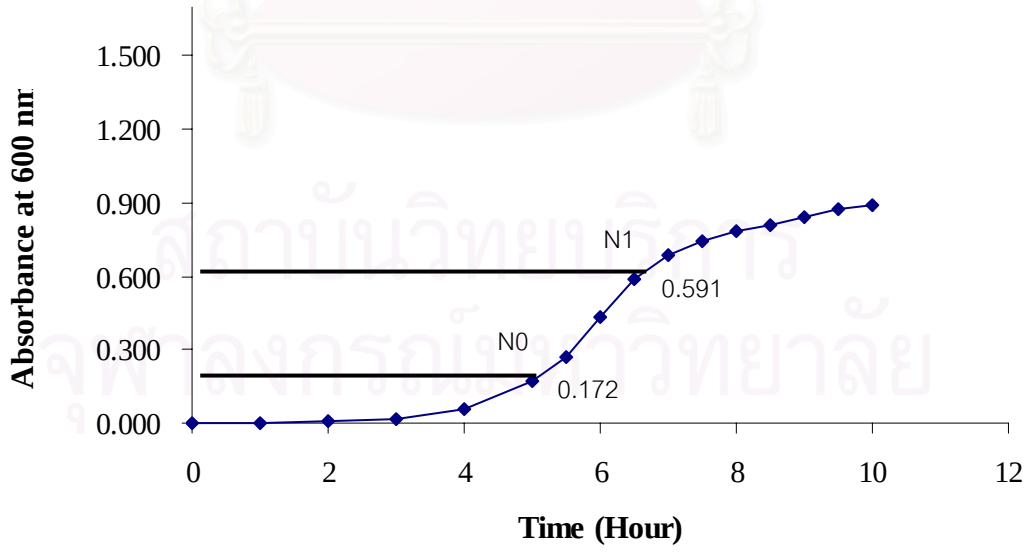
The values shown in the table were averaged from triplicate.

9. Growth curve of food-borne pathogenic bacteria and *Pediococcus pentosaceus* NSL13-2



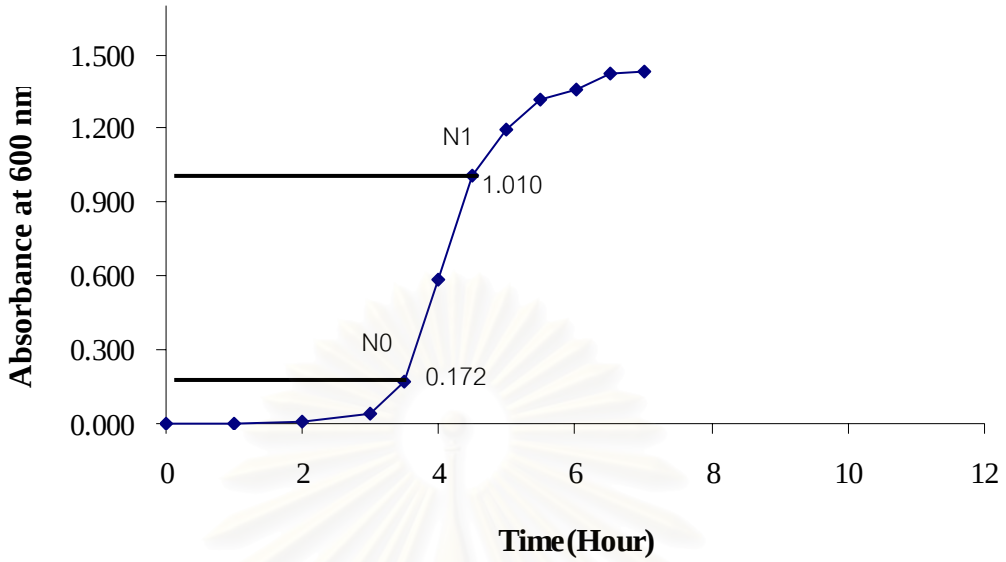
Doubling time of *Staphylococcus aureus* ATCC 25923 is 1 hour 4 minutes

Figure F1 Growth curve of *Staphylococcus aureus* ATCC 25923



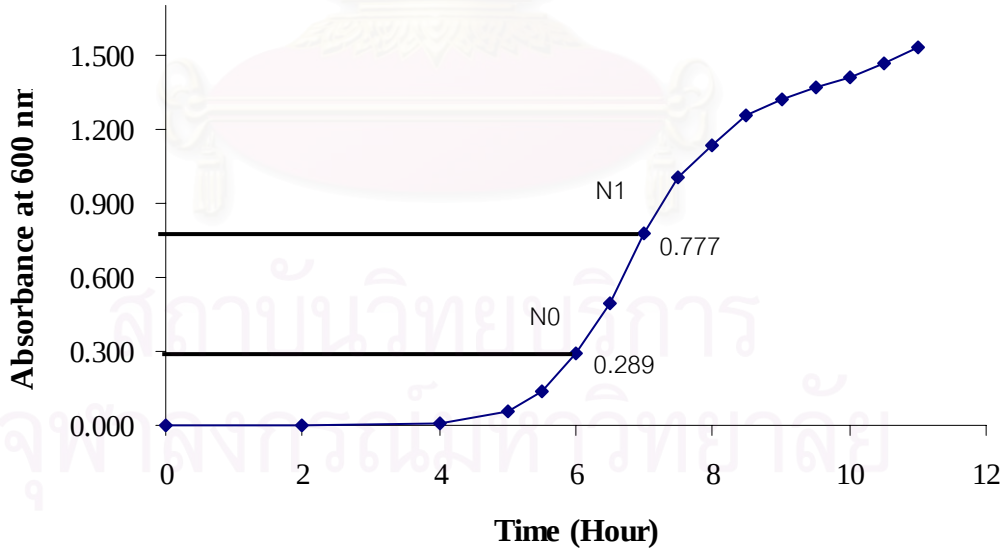
Doubling time of *Listeria monocytogenes* DMST 17303 is 51 minutes

Figure F2 Growth curve of *Listeria monocytogenes* DMST 17303



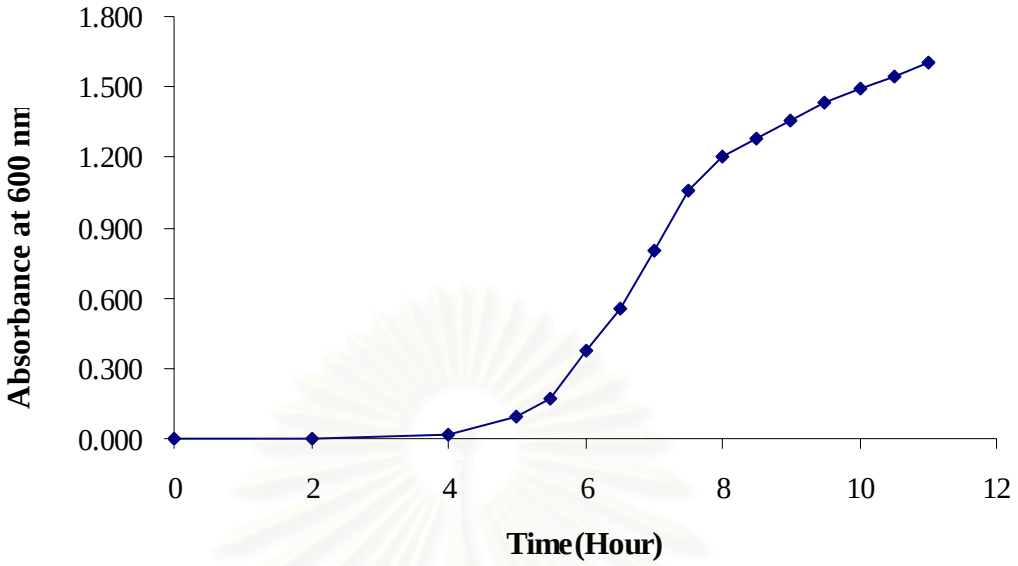
Doubling time of *Bacillus cereus* ATCC 1729 is 24 minutes

Figure F3 Growth curve of *Bacillus cereus* ATCC 1729



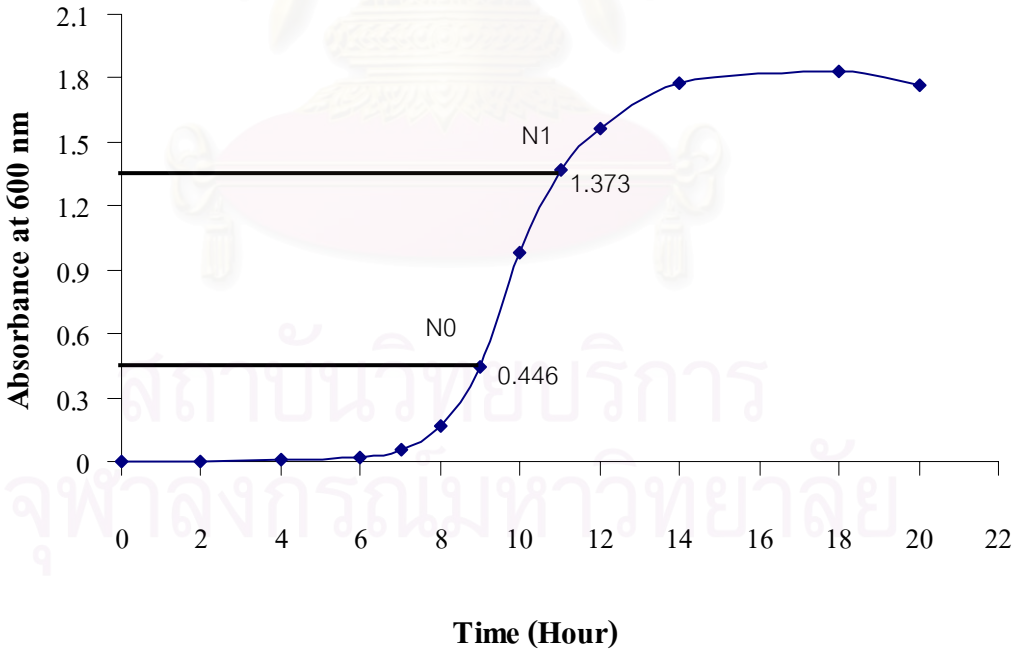
Doubling time of *Escherichia coli* ATCC 25922 is 42 minutes

Figure F4 Growth curve of *Escherichia coli* ATCC 25922



Doubling time of *Salmonella* Typhimurium ATCC 13311 is 55 minutes

Figure F5 Growth curve of *Salmonella* Typhimurium ATCC 13311



Doubling time of *Pediococcus pentosaceus* NSL13-2 is 1 hour 13 minutes

Figure F6 Growth curve of *Pediococcus pentosaceus* NSL13-2

10. Table F9 Viable counts of bacteria and pH measurement investigation of inhibitory efficiency of *Pediococcus pentosaceus* NSL 13-2 on *S. aureus* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL13-2 (control A)	NSL13-2 (co-culture)	*S. aureus* (control B)	*S. aureus* (co-culture)	
0	6.82	6.79	4.85	4.94	6.52
4	8.08	8.25	6.32	4.92	6.30
8	9.47	9.25	6.83	4.52	4.30
12	9.35	9.13	6.89	0	4.20
16	9.31	9.17	7.19	0	4.03
20	9.38	9.23	7.33	0	3.99
24	9.44	9.44	7.33	0	3.90
48	7.92	8.37	7.54	0	3.86

11. Table F10 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Enterococcus gilvus* NSL5-2 on *S. aureus* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL5-2 (control A)	NSL5-2 (co-culture)	*S. aureus* (control B)	*S. aureus* (co-culture)	
0	6.42	6.37	4.80	4.84	6.66
4	8.41	8.47	6.13	5.91	5.76
8	8.81	8.57	6.79	5.54	5.08
12	8.94	8.87	6.84	3.78	4.75
16	8.73	8.87	7.16	1.30	4.62
20	8.88	8.87	7.38	0	4.54
24	8.80	8.65	7.47	0	4.48
48	7.82	7.71	7.56	0	4.37

12. Table F11 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Weissella cibaria* NSL1-4 on *S. aureus* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL1-4 (control A)	NSL1-4 (co-culture)	*S. aureus* (control B)	*S. aureus* (co-culture)	
0	6.54	6.56	4.73	4.68	6.67
4	7.12	7.29	6.30	5.69	6.51
8	9.28	9.41	6.44	0	4.88
12	9.42	9.41	6.79	0	4.32
16	9.38	9.32	7.15	0	4.32
20	8.64	9.10	7.85	0	4.32
24	7.90	7.92	7.45	0	4.33
48	0.00	0.00	7.58	0	4.32

13. Table F12 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Pediococcus pentosaceus* NSL13-2 on *E. coli* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL13-2 (control A)	NSL13-2 (co-culture)	*E. coli* (control B)	*E. coli* (co-culture)	
0	6.62	6.57	4.81	4.80	6.57
4	8.28	8.26	5.19	5.59	6.35
8	9.48	9.41	6.70	5.74	4.45
12	9.45	9.42	8.62	3.52	4.04
16	9.61	9.44	8.65	0	3.97
20	9.55	9.45	8.62	0	3.88
24	9.53	9.39	8.76	0	3.90
48	9.12	7.95	8.33	0	3.82

14. Table F13 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Enterococcus gilvus* NSL5-2 on *E. coli* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL5-2 (control A)	NSL5-2 (co-culture)	*E. coli* (control B)	*E. coli* (co-culture)	
0	6.38	6.36	4.69	4.75	6.52
4	7.70	7.80	5.67	5.37	6.37
8	9.21	9.07	7.44	6.19	5.33
12	8.62	8.64	8.57	4.48	4.90
16	8.72	8.88	8.56	4.23	4.75
20	8.72	8.66	8.65	1.90	4.67
24	8.81	8.79	8.50	1.84	4.43
48	8.55	8.27	8.69	0	4.30

15. Table F14 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Weissella cibaria* NSL1-4 on *E. coli* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL1-4 (control A)	NSL1-4 (co-culture)	*E. coli* (control B)	*E. coli* (co-culture)	
0	6.55	6.56	4.70	4.66	6.58
4	9.23	9.17	5.24	4.00	5.03
8	9.28	9.21	6.90	0	4.37
12	9.41	9.34	8.50	0	4.32
16	9.44	9.39	8.73	0	4.30
20	9.36	9.36	8.70	0	4.27
24	7.51	7.54	8.59	0	4.29
48	0.00	0.00	8.64	0	4.29

16. Table F15 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Pediococcus pentosaceus* NSL13-2 on *L. monocytogenes* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL13-2 (control A)	NSL13-2 (co-culture)	*L.monocytogenes* (control B)	*L.monocytogenes* (co-culture)	
0	6.62	6.58	3.98	4.03	6.52
4	8.46	8.44	5.54	5.17	6.16
8	9.38	9.62	7.42	5.16	4.34
12	9.54	9.40	8.42	0	4.06
16	9.37	9.41	9.13	0	3.97
20	9.38	9.45	9.10	0	3.96
24	9.43	9.47	9.22	0	3.89
48	8.00	8.08	7.88	0	3.85

17. Table F16 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Pediococcus pentosaceus* NSL13-2 on *B. cereus* in MRS broth at different time

Time (hour)	Log CFUml^{-1}				pH (co-culture)
	NSL13-2 (control A)	NSL13-2 (co-culture)	*B. cereus* (control B)	*B.cereus* (co-culture)	
0	6.70	6.81	4.16	4.15	6.63
4	8.86	8.94	5.73	5.07	5.64
8	9.61	9.53	7.38	0	4.27
12	9.40	9.34	8.09	0	4.00
16	9.78	9.14	8.20	0	3.96
20	9.27	9.38	8.07	0	3.91
24	9.23	9.15	8.11	0	3.89
48	8.09	7.90	8.06	0	3.86

18. Table F17 Viable counts of bacteria and pH measurement during investigation of inhibitory efficiency of *Pediococcus pentosaceus* NSL13-2 on *S.* Typhimurium in MRS broth at different time

Time (hour)	Log $CFUml^{-1}$				pH (co-culture)
	NSL13-2 (control A)	NSL13-2 (co-culture)	*S.* Typhimurium (control B)	*S.* Typhimurium (co-culture)	
0	6.53	6.51	4.18	4.17	6.56
4	8.15	8.35	4.48	4.50	6.39
8	9.38	9.41	4.97	3.68	4.39
12	9.48	9.37	7.40	0	4.04
16	9.36	9.33	8.20	0	3.95
20	9.37	9.38	8.41	0	3.89
24	9.44	9.38	8.46	0	3.87
48	8.03	8.02	7.58	0	3.84

19. Table F18 Viable counts of bacteria, pH measurement and relative of total acid increase (%w/v) during the fermentation of nham inoculated with 10^6CFUml^{-1} *P. pentosaceus* NSL13-2 and 10^4 CFUml^{-1} *S. aureus*

Time (hour)	Log CFUml^{-1}			pH	Relative total acid increase (%w/v)
	LAB	Total bacteria count	*S.aurues*		
Nham control (NNF)					
0	5.83	6.25	2.52	5.73	ND
24	9.42	9.27	3.43	4.84	ND
72	9.41	9.14	3.75	4.46	ND
120	9.04	8.31	2.72	4.36	ND
168	8.71	7.79	4.01	4.35	ND
Nham + *P. pentosaceus* NSL13-2 (NS)					
0	6.82	6.43	2.41	5.72	0.45
24	9.10	8.44	2.59	4.83	0.63
72	9.16	8.42	2.45	4.42	0.83
120	9.12	7.89	0	4.33	1.06
168	8.99	7.52	0	4.30	1.24
Nham + *S. aureus* (NP)					
0	5.96	6.17	3.71	5.71	ND
24	9.21	9.21	4.96	4.80	ND
72	9.33	9.03	4.14	4.42	ND
120	8.72	8.25	3.38	4.35	ND
168	8.81	7.73	2.51	4.33	ND
Nham + *P. pentosaceus* NSL13-2 + *S. aureus* (NSP)					
0	6.90	6.11	3.68	5.69	0.47
24	9.18	8.54	3.70	4.80	0.58
72	9.02	8.90	2.45	4.37	0.86
120	8.96	7.76	0	4.28	1.01
168	8.63	7.34	0	4.28	1.15

ND = Not done

20. Table F19 Viable counts of bacteria, pH measurement and total acidity analysis during the fermentation of nham inoculated with 10^6 $CFUml^{-1}$ *P. pentosaceus* NSL13-2 and 10^4 $CFUml^{-1}$ *L. monocytogenes*

Time (hour)	Log $CFUml^{-1}$			pH	% Relative total acidity (%w/v)
	LAB	Total bacteria count	*L. monocytogenes*		
Nham control (NNF)					
0	5.15	5.28	0	5.84	ND
24	9.28	9.12	0	5.26	ND
72	8.99	8.63	0	4.59	ND
120	8.59	7.87	0	4.45	ND
168	8.27	5.67	0	4.35	ND
Nham + *P. pentosaceus* NSL13-2 (NS)					
0	6.76	5.07	0	5.80	0.50
24	9.15	8.59	0	4.99	0.60
72	8.96	7.82	0	4.52	0.77
120	8.61	6.72	0	4.40	0.84
168	8.36	6.19	0	4.39	0.99
Nham + *L. monocytogenes* (NP)					
0	5.31	5.38	4.25	5.82	ND
24	9.26	9.02	5.03	5.17	ND
72	8.95	8.67	4.85	4.53	ND
120	8.55	7.62	4.65	4.37	ND
168	7.77	5.62	4.08	4.32	ND
Nham + *P. pentosaceus* NSL13-2 + *L. monocytogenes* (NSP)					
0	6.64	5.31	4.10	5.60	0.49
24	8.97	8.06	4.87	5.03	0.57
72	8.84	7.83	4.57	4.57	0.80
120	8.68	6.32	3.78	4.44	0.92
168	8.35	5.57	2.26	4.42	1.01

ND = Not done

21. Table F20 Viable counts of bacteria, pH measurement and total acidity analysis during the fermentation of nham inoculated with 10^6 $CFUml^{-1}$ *P. pentosaceus* NSL13-2 and 10^4 $CFUml^{-1}$ *B. cereus*

Time (hour)	Log $CFUml^{-1}$			pH	% Relative total acidity (%w/v)
	LAB	Total bacteria count	*B. cereus*		
Nham control (NNF)					
0	4.68	5.04	0	5.61	ND
24	9.26	9.10	0	5.38	ND
72	9.34	8.54	0	4.57	ND
120	8.39	7.76	0	4.44	ND
168	8.12	6.97	0	4.39	ND
Nham + *P. pentosaceus* NSL13-2 (NS)					
0	6.94	4.98	0	5.61	0.47
24	9.17	7.95	0	4.94	0.64
72	9.24	7.54	0	4.50	0.82
120	8.69	6.70	0	4.42	0.99
168	8.41	6.64	0	4.41	1.14
Nham + *B. cereus* (NP)					
0	4.87	5.20	4.15	5.64	ND
24	9.33	9.18	0	5.31	ND
72	9.25	9.04	0	4.55	ND
120	8.42	7.71	0	4.46	ND
168	8.17	7.27	0	4.28	ND
Nham + *P. pentosaceus* NSL13-2 + *B. cereus* (NSP)					
0	6.97	4.90	4.15	5.58	0.49
24	9.25	7.76	0	4.93	0.67
72	9.24	7.18	0	4.55	0.82
120	8.69	7.45	0	4.44	0.99
168	8.45	6.60	0	4.40	1.14

ND = Not done

22. Table F21 Viable counts of bacteria, pH measurement and total acidity analysis during the fermentation of nham inoculated with 10^6 $CFUml^{-1}$ *P. pentosaceus* NSL13-2 and 10^4 $CFUml^{-1}$ *E. coli*

Time (hour)	Log $CFUml^{-1}$			pH	% Relative total acidity (%w/v)
	LAB	Total bacteria count	*E. coli*		
Nham control (NNF)					
0	5.39	5.46	0	5.58	ND
24	9.54	9.47	0	5.56	ND
72	9.29	9.05	0	4.84	ND
120	8.81	8.21	0	4.65	ND
168	8.73	7.15	0	4.56	ND
Nham + *P. pentosaceus* NSL13-2 (NS)					
0	6.83	5.55	0	5.58	0.47
24	9.35	8.39	0	5.03	0.59
72	9.28	8.05	0	4.56	0.69
120	8.72	6.99	0	4.46	0.89
168	8.58	6.47	0	4.39	0.99
Nham + *E. coli* (NP)					
0	5.74	5.76	4.65	5.63	ND
24	9.46	9.41	6.44	5.49	ND
72	9.27	8.95	6.14	4.79	ND
120	9.32	8.16	5.31	4.59	ND
168	8.73	7.24	4.62	4.54	ND
Nham + *P. pentosaceus* NSL13-2 + *E. coli* (NSP)					
0	6.82	5.96	4.75	5.60	0.49
24	9.47	8.20	6.31	5.03	0.57
72	9.26	7.80	5.47	4.57	0.80
120	9.32	6.95	3.67	4.44	0.92
168	8.70	6.92	3.23	4.42	1.01

ND = Not done

23. Table F22 Viable counts of bacteria, pH measurement and total acidity analysis during the fermentation of nham inoculated with 10^6 $CFUml^{-1}$ *P. pentosaceus* NSL13-2 and 10^4 $CFUml^{-1}$ *S.* Typhimurium

Time (hour)	Log $CFUml^{-1}$			pH	% Relative total acidity (%w/v)
	LAB	Total bacteria count	*S.* Typhimurium		
Nham control (NNF)					
0	5.03	5.89	0	5.56	ND
24	9.16	9.12	0	5.39	ND
72	8.71	8.77	0	4.86	ND
120	8.57	8.38	0	4.62	ND
168	8.41	7.99	0	4.61	ND
Nham + *P. pentosaceus* NSL13-2 (NS)					
0	6.88	5.88	0	5.55	0.57
24	9.00	8.19	0	4.94	0.77
72	9.07	8.06	0	4.49	0.89
120	8.56	7.24	0	4.39	1.14
168	8.35	6.94	0	4.37	1.21
Nham + *S.* Typhimurium (NP)					
0	5.04	5.72	4.31	5.54	ND
24	9.15	9.13	8.86	5.30	ND
72	8.72	8.93	8.56	4.79	ND
120	8.38	8.17	8.45	4.66	ND
168	8.14	7.75	8.17	4.52	ND
Nham + *P. pentosaceus* NSL13-2 + *S.* Typhimurium (NSP)					
0	6.75	5.75	4.30	5.53	0.59
24	9.00	8.05	8.44	4.89	0.69
72	8.82	6.78	9.07	4.47	0.92
120	9.33	6.85	0	4.41	1.09
168	8.21	6.92	0	4.34	1.21

ND = Not done

Appendix G

Nucleotide Sequences

1. Nucleotide sequences of NSL13-2 as followed:

5'- GATTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGG
GTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGAT
GCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTAT
CACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAG
GCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCT
GATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTA
AAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCG
GCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGT
GGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAA
GGCGGCTGTCTGGTCTGCACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGG
ATTAGATACCTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCC
GCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGCCTGGGAGTACGACCGCAAG
GTTGAAACTCAAAAGAATTGACGGGGCCCGCACAGCGGTGGAGCATGTGGTTTAATT
CGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTA
GAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCAT
TAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGA
CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC
AACGAGTCGCGAGACCGCGAGGTTAAGCTAATCTCTTAAAACCATTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCAT

GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTT
TGTAACACCCAAAGCCGGTGGGGTAACCTTTTAGGAGCTAGCCGTCTAAGG-3'

2.Nucleotide of the partial 16S rRNA gene of *Pediococcus pentosaceus* strain RTa11, the database sequence for comparing with NSL13-2

LOCUS: FM179610; 1564 bp DNA linear

DEFINITION: *Pediococcus pentosaceus* partial 16S rRNA gene, strain RTa11.

ACCESSION: FM179610

REFERENCE 2 (bases 1 to 1564)

AUTHORS: Schmidt, H.

TITLE: Direct Submission

JOURNAL: Submitted (07-JUL-2008) Schmidt H., Food Microbiology, University of Hohenheim, Garbenstrasse 28, Stuttgart, 70599, GERMANY

ORIGIN

1 agtttgatta tggctcagga tgaacgctgg cggcgtgcct aatacatgca agtcgaacga
61 acttccgtta attgattatg acgtacttgt actgattgag attttaacac gaagtgagtg
121 gcgaacgggt gagtaacacg tgggtaacct gcccagaagt aggggataac acctggaaac
181 agatgctaat accgtataac agagaaaacc gcatggtttt cttttaaaag atggctctgc
241 tatcacttct ggatggaccc gcggcgtatt agctagttgg tgaggtaaag gctcaccaag
301 gcagtgatac gtagccgacc tgagagggta atcggccaca ttgggactga gacacggccc
361 agactcctac gggaggcagc agtagggaat cttccacaat ggacgcaagt ctgatggagc
421 aacgccgcgt gagtgaagaa gggtttcggc tcgtaaagct ctgttgttaa agaagaacgt
481 gggtaagagt aactgtttac ccagtgacgg tatttaacca gaaagccacg gctaactacg
541 tgccagcagc cgcggtaata cgtaggtggc aagcgttatc cggatttatt gggcgtaaag
601 cgagcgcagg cggtctttta agtctaatgt gaaagccttc ggctcaaccg aagaagtgca
661 ttggaaactg ggagacttga gtgcagaaga ggacagtgga actccatgtg tagcggtgaa
721 atgcgtagat atatggaaga acaccagtgg cgaaggcggc tgtctggtct gcaactgacg
781 ctgaggctcg aaagcatggg tagcgaacag gattagatac cctggtagtc catgccgtaa

841 acgatgatta ctaagtgttg gagggtttcc gcccttcagt gctgcagcta acgcattaag
901 taatccgcct ggggagtacg accgcaaggt tgaaactcaa aagaattgac gggggcccgc
961 acaagcggtg gagcatgtgg tttaattcga agctacgcga agaaccttac caggtcttga
1021 catcttctga cagtctaaga gattagaggt tcccttcggg gacagaatga caggtggtgc
1081 atggttgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc
1141 ttattactag ttgccagcat taagttgggc actctagtga gactgccggt gacaaaccgg
1201 aggaaggtgg ggacgacgtc aaatcatcat gccccttatg acctgggcta cacacgtgct
1261 acaatggatg gtacaacgag tcgcgagacc gcgaggttaa gctaatctct taaaaccatt
1321 ctcagttcgg actgtaggct gcaactcgcc tacacgaagt cggaatcgct agtaatcgcg
1381 gatcagcatg ccgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacaccatg
1441 agagtttgta acacccaaag ccggtggggt aacctttag gagctagccg tctaaggtgg
1501 gacagatgat tagggtgaag tcgtaacaag gtagccgtag gagaacctgc ggctggatca
1561 cctc

3.Nucleotide sequences of NSL1-4 as followed:

5’-TGCAAGTCGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGCTCAGATATGACG
ATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTA
GCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATG
GTTGCTACTTAAAAGATGGTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGTT
AGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGA
TCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGT
TTCGGCTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGT
GTGACGGTATCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATA
CGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATT
TAAGTCTGAAGTGAAAGCCTCAGCTCACTGAGGAATTGCTTTGGAAACTGGATGACTT
GAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATG
GAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAA
AGTGTGGGTACAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTG

CTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAGCACTCCG
CCTGGGGAGTACGACCGCAAGGTGAAACTCAAAGGAATTGACGGGGACCCGCACAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTACTAGTTGCCAGCATTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCT
CTTAAAGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAAT
CGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACA
CCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCTTCGG
GAGCCAGCCGTCTAAGG-3’

4. Nucleotide of the partial 16S rRNA gene of *Weissella cibaria* strain NRIC 0136, the database sequence for comparing with NSL1-4

LOCUS: AB362617; 1570 bp DNA linear
DEFINITION: *Weissella cibaria* gene for 16S rRNA, partial sequence, strain: NRIC 0136.
ACCESSION: AB362617
REFERENCE: 2 (bases 1 to 1570)
AUTHORS: Tanaka, N., Nakano, M. and Okada, S.
TITLE: Direct Submission
JOURNAL: Submitted (01-OCT-2007) Naoto Tanaka, Tokyo University of Agriculture, NODAI Culture Collection Center; 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan (E-mail:n3tanaka@nodai.ac.jp, Tel:81-3-5477-2725, Fax:81-3-5477-2537)

ORIGIN

1 agagtttgat catggctcag gatgaacgct ggcggcgtgc ctaatacatg caagtcgaac
61 gctttgtggt tcaactgatt tgaagagctt gctcagatat gacgatggac attgcaaaga
121 gtggcgaacg ggtgagtaac acgtgggaaa cctacctctt agcaggggat aacatttgga
181 aacagatgct aataccgtat aacaatagca accgcatggt tgctacttaa aagatggttc
241 tgctatcact aagagatggt cccgcggtgc attagttagt tggtgaggta atggctcacc
301 aagacgatga tgcatagccg agttgagaga ctgatcggcc acaatgggac tgagacacgg
361 cccatactcc tacgggaggc agcagtaggg aatcttccac aatgggcgaa agcctgatgg
421 agcaacgccg cgtgtgtgat gaagggtttc ggctcgtaaa acactgttgt aagagaagaa
481 tgacattgag agtaactgtt caatgtgtga cggtatctta ccagaaagga acggctaaat
541 acgtgccagc agccgcggta atacgtatgt tccaagcgtt atccggattt attgggcgta
601 aagcgagcgc agacggttat ttaagtctga agtgaaagcc ctcagctcaa ctgaggaatt
661 gctttggaaa ctggatgact tgagtgcagt agaggaaagt ggaactccat gtgtagcggt
721 gaaatgcgta gatatatgga agaacaccag tggcgaaggc ggctttctgg actgtaactg
781 acgttgaggc tcgaaagtgt gggtagcaaa caggattaga taccctggta gtccacaccg
841 taaacgatga gtgctaggtg tttgagggtt tccgccctta agtgccgcag ctaacgcatt
901 aagcactccg cctggggagt acgaccgcaa ggttgaaact caaaggaatt gacggggacc
961 cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg cgaagaacct taccaggtct
1021 tgacatccct tgacaactcc agagatggag cgttcccttc ggggacaagg tgacaggtgg
1081 tgcatggttg tcgtcagctc gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa
1141 cccttattac tagttgccag catttagttg ggcactctag tgagactgcc ggtgacaaac
1201 cggaggaagg tggggatgac gtcaaatcat catgcccctt atgacctggg ctacacacgt
1261 gctacaatgg cgtatacaac gagttgccaa cccgcgaggg tgagctaatc tcttaaagta
1321 cgtctcagtt cggattgtag gctgcaactc gcctacatga agtcggaatc gctagtaatc
1381 gcggatcagc acgccgcggt gaatacgttc ccgggtcttg tacacaccgc ccgtcacacc
1441 atgagagttt gtaacaccca aagccggtgg ggtaaccttc gggagccagc cgtctaaggt
1501 gggacagatg attagggtga agtcgtaaca aggtagccgt aggagaacct gcggttggat
1561 cacctcctta

5. Nucleotide sequences of NSL5-2 as followed:

5’- GAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA
CACTTGGAAACAGGTGCTAATACCGTATAACAATAGAAACCGCATGGTTTCTATTTGAA
AGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCA
ATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA
AAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTAT
CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTG
ATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGG
AACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCATGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGG
CTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT
GAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAG
GTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGCATAGATGA-3’

6. Nucleotide of the partial 16S rRNA gene of *Enterococcus gilvus* strain 2366, the database sequence for comparing with NSL5-2

LOCUS: EF535229; 1471 bp DNA linear

DEFINITION: *Enterococcus gilvus* strain 2366 16S ribosomal RNA gene, partial sequence.

ACCESSION: EF535229

REFERENCE 2 (bases 1 to 1471)

AUTHORS: Zago, M., Bonvini, B., Rossetti, L., Fornasari, M. E., Carminati, D. and Giraffa, G.

TITLE: Direct Submission

JOURNAL: Submitted (03-APR-2007) Microbiology, CRA-Istituto Sperimentale Lattiero-Caseario, via Lombardo, 11, Lodi, LO 26900, Italy

ORIGIN

1 cctggctcag gacgaacgct ggcggcgtgc ctaatacatg caagtcgaac gctttttctt
61 tcaccggagc ttgctccacc gaaagaaaag gagtggcgaa cgggtgagta acacgtgggt
121 aacctgccca tcagaagggg ataacacttg gaaacaggtg ctaataccgt ataacaatag
181 aaaccgcatg gtttctattt gaaaggcgct tttgcgtcac tgatggatgg acccgcggtg
241 cattagctag ttggtgaggt aacggctcac caaggcaacg atgcatagcc gacctgagag
301 ggtgatcggc cacattggga ctgagacacg gcccaaactc ctacgggagg cagcagtagg
361 gaatcttcgg caatggacga aagtctgacc gagcaacgcc gcgtgagtga agaaggtttt
421 cggatcgtaa aactctgttg ttagagaaga acaaggatga gagtaaaatg ttcatccctt
481 gacggtatct aaccagaaag ccacggctaa ctacgtgcca gcagccgcgg taatacgtag
541 gtggcaagcg ttgtccggat ttattgggcg taaagcgagc gcaggcggtt tcttaagtct
601 gatgtgaaag cccccggctc aaccggggag ggtcattgga aactgggaaa cttgagtgca
661 gaagaggaga gtggaattcc atgtgtagcg gtgaaatgcg tagatatatg gaggaacacc
721 agtggcgaag gcggctctct ggtctgtaac tgacgctgag gctcgaaagc gtggggagca
781 aacaggatta gataccctgg tagtccacgc cgtaaacgat gagtgctaag tgttggaggg
841 tttccgccct tcagtgctgc agctaacgca ttaagcactc cgcctgggga gtacgaccgc

901 aaggttgaaa ctcaaaggaa ttgacggggg cccgcacaag cggtggagca tgtggtttaa

961 ttcgaagcaa cgcgaagaac cttaccaggt cttgacatcc tttgaccact ctagagatag

1021 agcttcccct tcgggggcaa agtgacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg

1081 agatgttggg ttaagtcccg caacgagcgc aacccttatt gttagttgcc atcatttagt

1141 tgggcactct agcgagactg ccggtgacaa accggaggaa ggtggggatg acgtcaaatc

1201 atcatgcccc ttatgacctg ggctacacac gtgctacaat gggaagtaca acgagtcgcg

1261 aagtcgcgag gctaagctaa tctcttaaag cttctctcag ttcggattgt aggctgcaac

1321 tcgcctacat gaagccggaa tcgctagtaa tcgcggatca gcacgccgcg gtgaatacgt

1381 tcccgggcct tgtacacacc gcccgtcaca ccacgagagt ttgtaacacc cgaagtcggt

1441 gaggtaacct tttggagcca gccgcctaag g

BIOGRAPHY

Miss Kanchaniga Rungreangsuk was born on November 18, 1983 in Bangkok province. She graduated a Bachelor's degree of Science (2nd class honours) majoring in Biotechnology from King Mongkut's Institute of Technology Ladkrabang in 2006. She continued her study in a Master's degree in Faculty of Science majoring in Industrial Microbiology in Chulalongkorn University and expected to finish by the academic year of 2008.

Academic Presentation

Kanchaniga Rungreangsuk and Sirirat Rengpipat. Selection of bacteriocin-producing lactic acid bacteria from Nham for inhibition of pathogenic bacteria. Proceedings in the 20th Annual Meeting and International Conference of the Thai Society for Biotechnology (TSB). October14-17, 2008, Mahasarakham University, Mahasarakham, Thailand. p. 150 (poster presentation)(Full text in CD-ROM)

Research Fund

CU Graduate School Thesis Grant, Chulalongkorn University

Teaching Assistance in Department of Microbiology, Faculty of Science, Chulalongkorn University

Academic year 2007 and 2008

2312513 Bacterial Systematics

2312541 Medical Bacteriology