Value Enhancement of Fermented Ground Pork Product Using γ-Aminobutyric Acid Producing Starter Lactic Acid Bacteria

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การเพิ่มคุณค่าผลิตภัณฑ์แหนมโดยใช้กล้าเชื้อแลกติกแอสิดแบคทีเรียที่ผลิตกาบา (γ-aminobutyric acid)

ผู้เขียน นางสาวอนุสรา รัตนบุรี

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บทคัดย่อ

เพื่อเพิ่มมูลค่าผลิตภัณฑ์อาหารเนื้อหมักของไทย เช่น แหนม ให้ส่งผลดีต่อสุขภาพ จึงได้คัดแยกแลกติกแอสิดแบคทีเรียจำนวน 602 ไอโซเลท จากตัวอย่างอาหารหมักชนิดต่างๆ และคัดเลือกเชื้อมาจำนวน 14 ไอโซเลท ที่สามารถผลิตกาบา (γ-aminobutyric acid; GABA) ได้สูงพร้อมกับทดสอบความสามารถในการยับยั้งแบคทีเรียก่อโรคของระบบทางเดินอาหารที่เลือกใช้เป็นอินดิเคเตอร์ 7 ชนิด (*Bacillus cereus* ATCC 11778 *Clostridium perfringens* DMST 16637 *Listeria monocytogenes* DMST 4553 *Staphylococcus aureus* ATCC 25923 *Escherichia coli* ATCC 25922 *Salmonella Typhi* PSSCMI 0034 และ *Shigella sonneii* PSSCMI 0032) ซึ่งคัดเลือกเชื้อได้จำนวน 4 ไอโซเลทที่ผลิตกาบาในช่วง 7,339–9,060 มิลลิกรัมต่อลิตร โดยลำดับการผลิตกาบาของไอโซเลท NH2 < NH102 < NH116 < HN8 และพีเอชในน้ำเลี้ยงเซลล์มีค่า 3.8 ยกเว้น ไอโซเลท NH2 เป็น 4.2 ซึ่งทุกไอโซเลทสามารถยับยั้งเชื้อทดสอบได้ทุกชนิด เมื่อนำไปทดสอบคุณสมบัติการเป็นโปรไบโอติกโดยอิงคุณสมบัติทางด้านสรีรวิทยาและหน้าที่ของเชื้อ พบว่าไอโซเลท NH102 มีความสามารถในการทนต่อสภาวะเลียนแบบในกระเพาะอาหารและลำไส้เล็กได้ดีที่สุด คือเมื่อผ่านไป 4 ชั่วโมง เซลล์ลดลงเพียง 1 log CFU/mL ขณะที่ไอโซเลท NH116 มีความสามารถลดคอเลสเตอรอลได้ 43% นอกจากนี้ทุกไอโซเลทสามารถย่อยเกลือน้ำดีและไม่ย่อยเม็ดเลือดแดง เมื่อนำเชื้อดังกล่าวไปเทียบเคียงชนิด พบว่าไอโซเลท NH2 คือ *Lactobacillus namurensis* ส่วนไอโซเลทอื่นๆ คือ *Pediococcus pentosaceus* โดยที่ไอโซเลท NH2 และ HN8 มีศักยภาพในการเป็นกล้าเชื้อสำหรับการผลิตผลิตภัณฑ์เนื้อหมัก เมื่อนำกล้าเชื้อทั้ง 2 ชนิดมาผลิตแหนมที่อุดมด้วยสารกาบา

(GABA) โดยการออกแบบที่ใช้ central composite design (CCD) พบว่าการเติม 0.5% ผงชูรส (monosodium glutamate: MSG) และกล้าเชื้อ (แต่ละชนิดประมาณ 6 log CFU/g) พบว่าแหนมที่ได้มีกาบาสูงถึง 4,051 มิลลิกรัมต่อกิโลกรัม ซึ่งสูงกว่าแหนมที่มีชื่อทางการค้าและเป็นที่นิยมถึง 8 เท่า โดยแหนมกาบาคือแหนมที่เติมทั้งกล้าเชื้อและผงชูรส (T_{SM}) ทำให้มีแลกติก-แอสิดแบคทีเรียสูงที่สุด ส่วนยีสต์ต่ำสุด และไม่พบ staphylococci รวมทั้งเชื้อรา เมื่อเปรียบเทียบกับชุดควบคุมที่ไม่เติมอะไรเลย (T_{NN}) หรือเติมเพียงผงชูรส (T_{NM}) หรือเติมเพียงกล้าเชื้อ (T_{SN}) การวิเคราะห์ปริมาณสารอาหารหลักพบว่าแหนมกาบามีปริมาณไขมัน คาร์โบไฮเดรต และพลังงานต่ำ แม้ว่าเนื้อสัมผัสและสีมีความแตกต่างเล็กน้อยจากชุดควบคุม (T_{NN}) อย่างไรก็ตามผลการทดสอบทางประสาทสัมผัสพบว่าแหนมกาบาได้รับการยอมรับมากกว่าชุดควบคุม และแหนมทางการค้าในทุกพารามิเตอร์ที่ทดสอบ นอกจากนี้พบว่าแหนมกาบามีปริมาณไบโอเจนิกเอมีน 7 ชนิดที่ตรวจสอบผ่านเกณฑ์มาตรฐานที่กำหนด โดยความสามารถที่ลดจากมากไปน้อยดังนี้ β-phenylethylamine histamine tyramine putrescine spermine spermidine และ cadaverine ขณะที่แหนมชุดควบคุม (ไม่เติมกล้าเชื้อ: T_{NN} และ T_{NM}) มีปริมาณฮีสตามินเกินเกณฑ์ที่กำหนด ขณะที่ปริมาณโคเลสเตอรอลของแหนมกาบา (กาบา 3,962 มิลลิกรัมต่อกิโลกรัม) ลดลง 35% โดยมี 96 มิลลิกรัมต่อหนึ่งร้อยกรัม เมื่อเทียบกับชุดควบคุม (T_{NN}) และต่ำกว่าแหนมทางการค้า (114-125 มิลลิกรัมต่อหนึ่งร้อยกรัม) อย่างมีนัยสำคัญทางสถิติ สำหรับการติดตามการเปลี่ยนแปลงของแลกติกแอสิดแบคทีเรียในการหมักแหนมเป็นเวลา 4 วัน ด้วยวิธีการดั้งเดิม (โดยใช้ตัวแทนกลุ่ม 286 ไอโซเลท) และเทคนิคดีจีจีอี (Denaturing gradient gel electrophoresis) พบแลกติกแอสิดแบคทีเรีย 11 ชนิด ดังนี้ *L. plantarum L. brevis L. curvatus L. farciminis L. namurensis L. rhamnosus L. sake Lactococcus lactis P. acidilactici P. pentosaceus* และ *Weissella viridescens* โดยเชื้อหลักที่พบตลอดการหมักได้แก่ *L. namurensis* และ *P. pentosaceus* ซึ่งเป็นสายพันธุ์เดียวกับกล้าเชื้อที่เติมลงไปในสูตรแหนมกาบาเป็นการยืนยันว่ากล้าเชื้อที่ใช้มีบทบาทที่สำคัญทำให้ไบโอเจนิกเอมีนและโคเลสเตอรอลลดลง ดังนั้นการใช้กล้าเชื้อผสมในการผลิตไส้กรอกหมูหมักหรือแหนมของไทยได้รับการพัฒนาประสบความสำเร็จโดยได้แหนมที่อุดมด้วยสารกาบาที่มีปริมาณไบโอเจนิกเอมีนและโคเลสเตอรอลต่ำ

คำสำคัญ: กล้าเชื้อ, กาบา, เนื้อหมัก, แลกติกแอสิดแบคทีเรีย, วิธีการตอบสนองพื้นผิว, แหนม

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ABSTRACT

In order to produce a novel product of Thai fermented meat, such as healthy Nham, 602 lactic acid bacteria (LAB) isolates were isolated from various fermented foods and 14 isolates that produced high amounts of γ-aminobutyric acid (GABA) were also investigated for inhibition activity against seven food-borne bacteria (*Bacillus cereus* ATCC 11778, *Clostridium perfringens* DMST 16637, *Listeria monocytogenes* DMST 4553, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella Typhi* PSSCMI 0034 and *Shigella sonneii* PSSCMI 0032). Four selected isolates produced GABA in the range of 7,339–9,060 mg/L by NH2 < NH102 < NH116 < HN8; and pH of their culture filtrates was 3.8, except 4.2 for strain NH2, All 4 isolates inhibited all target organisms. Selected strains were further investigated for their physiological and functional properties of probiotics *in vitro*. Strain NH102 survived best in the gastrointestinal tract with only a one log cell decrease over 4 h, while strain NH116 was the best for cholesterol removal (43%). None of them showed any haemolysis and all selected strains hydrolased bile salts. Strain NH2 was identified as *Lactobacillus namurensis* and all the other strains were *Pediococcus pentosaceus*. Strains HN8 and NH2 were potential starters for GABA Nham fermentation. The use of central composite design (CCD) showed that addition of 0.5% monosodium glutamate (MSG) together with an inoculum size of roughly 6 log CFU/g of each of the two strains produced a maximal amounts of GABA (4,051 mg/kg) in the GABA Nham product. This was higher than any current popular commercial Nham product by roughly 8 times. GABA Nham

with the additions of both starters and MSG (T_{SM}) supported maximum populations of LAB with a minimum of yeasts and no staphylococci or molds when compared to the controls that had no addition of any starter or MSG (T_{NN}), or only the addition of MSG (T_{NM}), or with only the starter (T_{SN}). Based on proximate analysis of all Nham sets, GABA Nham was low in fat, carbohydrate and energy although its texture and color was slightly different from the control (T_{NN}). However, sensory evaluations of GABA Nham were more acceptable than the controls and commercial Nham products for all tested parameters. In addition, GABA Nham had lower amounts of the 7 biogenic amines (BAs) to meet the safety regulation requirements by reducing BAs in the order of β-phenylethylamine > histamine > tyramine > putrescine > spermine > spermidine > cadaverine. In contrast, with a control fermentation in the absence of starter (T_{NN}, no starter + no MSG and T_{NM}, no starter + 0.5% MSG) histamine was higher than the recommended level. The cholesterol content was also reduced by 35% in GABA Nham (T_{SM}, GABA 3,962 mg/kg) to 96 mg/100 g when compared with a non inoculated fermentation (T_{NN}) and this was significantly lower than popular commercial Nham brands (114-125 mg/100 g). Succession of LAB in Nham fermentations for 4 days was investigated using the conventional method on the basis of 286 representative isolates, and denaturing gradient gel electrophoresis (DGGE) technique; the following detected LAB were *L. plantarum* , *L. brevis, L. curvatus, L. farciminis, L. namurensis, L. rhamnosus, L. sake, Lactococcus lactis, P. acidilactici, P. pentosaceus* and *Weissella viridescens*. The dominant LAB found throughout fermentations was *L. namurensis* and *P. pentosaceus*; and they were the same accession numbers with a mixed starter culture. This could confirm that the starter cultures used played the biggest roles in reducing both the BAs and cholesterol levels. Hence, a unique novel fermented pork sausage or Nham (high in GABA but low in BAs and cholesterol) has been successfully developed with the use of a mixed starter culture.

Keywords: γ-aminobutyric acid (GABA), fermented meat sausage, lactic acid bacteria, Nham, probiotics, starter cultures

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Anussara Ratanaburee

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LIST OF ABBREVITIONS

Abbreviations used throughout this thesis are listed below.

BAs	=	Biogenic amines
bp	=	Base pair(s)
◦C	=	Degree Celsius
cm	=	Centimetre (s)
DGGE		Denaturing gradient gel electrophoresis
GABA	=	γ-aminobutyric acid
g	=	Gram
HPLC	=	High Performance Liquid Chromatography
h	=	Hour
kb	=	Kilo base pair
LAB	=	Lactic acid bacteria
M	=	Molar
mA	=	Milli-ampere
min	=	Minute
mg/mL	=	Milligram per millilitre
mL	=	Millilitre
mM	=	Millimolar
PCR	=	Polymerase chain reaction
rpm	=	Round per minute
U	=	Unit
Vol.	=	Volume
v/v	=	Volume per volume
w/v	=	Weight per volume
w/w	=	Weight per weight
µg	=	Microgram
µg/µL	=	Microgram per microlitre

μg	=	Microgram
μL	=	Microlitre
μM	=	Micromolar
%	=	Percent

CHAPTER 1

INTRODUCTION

Rationale and Background

Traditional Thai food fermentation is one of the oldest biotechnological processes. It not only extends the shelf-life but also enhances the flavor, texture and nutritional quality of the products. Among a variety of Thai fermented meat products, Nham or fermented ground pork sausage is a popular product consumed throughout Thailand and this product is characterized by high acidity as the final pH of approximately 4.2-4.6 (Valyasevi and Rolle, 2002). Fermentation of Nham is a well-known microbial process and ecological studied during fermentation was dated back to the 1970s (Kunawasen, 2000). The dominant microorganisms are lactic acid bacteria (LAB) such as lactobacilli (*Lactobacillus. acidophilus*, *L. cellubiose*, *L. graminis*, *L. plantarum*, *L. pentosus*, *L. curvatus*, *L. sakei*, *L. delbruckei*, *L. paracasei* and *L. brevis*) and pediococci (*Pediococcus acidilactici* and *P. pentosaceus*) (Tanasupawat and Komagata, 1995; Valyasevi and Rolle, 2002). Other microorganisms, *Micrococcus* sp. and *Staphylococcus* sp. that are capable of reducing nitrate to nitrite and ensure color development were also detected (Wiriyacharee et al., 1995a). Nham production as household product relies on spontaneous Nham fermentation or naturally occurring LAB; therefore the quality of product can vary considerably. On the other hand, starter cultures have been used to produce Nham in a big scale production by some factories. In addition, spontaneous fermentation may produce fermented meat products with biogenic amines (BAs) due to acidification and proteolysis by microbial activities during meat fermentation (Bover-Cid et al., 2001). The consumption of foods containing high amounts of BAs can cause various diseases such as headaches, nausea, increased blood pressure, cardiac palpitations, etc.; this also includes their ability to react with nitrite to form nitrosamines, that are potentially carcinogenic, (Shalaby, 1996). Hence, the use of starter cultures may reduce BAs in fermented meat products.

Starter cultures are widely used for producing various kinds of fermented meat products including Nham to shorten the fermentation time, ensure proper acid production, helping color development, enhance the flavor, drip loss improvement, and inhibition of undesirable microorganisms (Kongtong, 2008). The development of starter cultures should involve intense research on the roles of microorganisms during the fermentation. The biochemical and physiological roles of these microorganisms in the development of flavor and aroma of the Nham product must be elucidated (Smitinont et al., 1999). This information can be considered as the criteria in the selection of starter microorganism for used in the fermentation. The starter cultures finally selected would be the one giving a satisfactory performance in the process and also giving an acceptable organoleptic evaluation of the Nham product. The development of starter cultures for Nham fermentation was initiated in the 1975 (Techapinyawat, 1975). The first commercial Nham production using starter culture technology has been successfully used by Wanasanun Co. Ltd, Thailand since 1990. Furthermore, developments of starter formula for Nham have been carried out by Valyasevi et al., (2001) to improve the quality of the product. Selected isolates from the dominant microorganism in good quality Nham were evaluated for their ability to use as starter culture for Nham fermentation as well as the sensory quality of the final product.

Over a few past decades, many studies have been focused on the effect of using starter culture on organoleptic properties, sensorial characteristic, biochemical and physico-chemical properties, and the food-borne pathogens of Nham (Petchsing and Woodburn, 1990, Svetvivadhana, 1990, Valyasevi et al., 2001, Kwanmuang, 2003, Visessanguan et al., 2004, 2006a). There is a little work concern to use starter cultures in fermented meat products for providing extra health benefits to consumers; however, the effect of using γ-aminobutyric acid (GABA) producing LAB as starter cultures for fermented plant beverages and dairy products have been extensively studied (Park and Oh, 2007; Kim et al., 2009; Ratanaburee et al., 2011a). It is well recognized that GABA is produced from glutamic acid or monosodium glutamate (MSG) by the catalytic activity of glutamate decarboxylase. MSG can dissociate into Na^+ and L-glutamic acid, which is the substrate for GABA synthesis (Higuchi et al.,

1997; Lu et al., 2009). Administration of GABA-enriched food could provide health benefits as it is effective for regulation of depression, sleeplessness, autonomic disorders and prevention of diabetic conditions (Siragusa et al., 2007). As there have not been reported of this we thought it of interest to try to produce a Nham product that enriched with GABA. In addition, to our knowledge, this is the first time that GABA-producing LAB has been applied as starter cultures for the fermentation of Nham products. Many factors such as amounts of MSG, NaCl and inoculum size are likely to affect on GABA content in Nham including the sensory of Nham product. Therefore to increase amount of GABA in Nham, those variable factors must be optimized to obtain Nham that enriched with GABA under acceptable level of consumers. A statistical experimental approach for optimizing those variables involves the application of concepts termed response surface methodology (RSM) using a central composite design (CCD) (Sadik et al., 2007; Tung et al., 2011).

The ability of probiotics to prevent diseases and improve health at all ages are increasing the market potential at a high rate (Bhadoria and Mahapatra, 2011); and LAB are well recognized for their abilities to be probiotics. Most of probiotic LAB are applied in dairy products; however, a numerous research has been focused to use probiotic LAB in fermented meat products. For instance, Erkkila et al., (2001) reported that the potentially probiotic strains; *L. rhamnosus* strains GG E-97800 and LC-705 are applicable for dry sausage fermented to enhance product safety while Ruiz-Moyano et al., (2011) demonstrated the usefulness of the potential probiotics of *L. reuteri* PL519 in a traditional Iberian dry fermented sausage. However, no work has been done to reduce cholesterol in fermented meat products although a lot of work focuses to study the role of probiotics to reduce cholesterol i.e. *L. plantarum* KLDS 1.0344 and *L. plantarum* TGCM 15 could lower the cholesterol content in culture media or fermented plant beverages (Guo et al., 2011; Silirun et al., 2010). In addition, several research groups have demonstrated that BAs can be decreased in fermented products especially meats by using an appropriate starter culture like probiotics (Latorre-Moratalla et al., 2012; Tosukhowong et al., 2011).

The study of the effect of the starter culture on LAB ecology in the fermentation processes are crucial since the information will allow a better

understanding of starter culture's role in Nham ecosystem for increasing the confidence of manufacture to decide the use of this microorganism in Nham production. Sensitive and reliable of detection and identification methods are important for monitoring population changes of both natural and starter cultures used in the fermentation process. Traditional methods for bacterial identification based on several properties; morphological, physiological and biochemical characteristics are laborious, time consuming, sensitive to growth conditions for expression of the characteristics, and often lacking sensitivity to detect small differences among closely related strains. Recently, molecular technique based on detection of differences in the genetic materials is an alternative method for a faster and more reliable microbial differentiation. Furthermore, many genotypic methods which are based on the principle of polymerase chain reaction (PCR) has been recognized as a simple PCR based technique with rapid, high discriminatory power, suitable for high through put analysis, and provide reliable results. The technique has gained popularity and is widely used in molecular typing of wide range of bacteria. Among molecular techniques, PCR of 16S rRNA genes based typing techniques, has been used to identify each bacterial isolates while Denaturing Gradient Gel Electrophoresis (DGGE) has been used to monitor changes of bacterial strains that present in community. According to above information the aims of this study are provided as follows.

Objectives

1. To isolate and select GABA producing LAB with potential to have probiotic properties for adding value to Nham,

2. To optimize conditions for producing GABA Nham on the basis of using starter cultures and adding monosodium glutamate,

3. To study microbial succession by focusing on lactic acid bacteria in spontaneous and inoculated Nham fermentations.

Scope of the study

LAB strains were isolated from various fermented foods to select the strains that can be used as starter cultures for producing a novel Nham product by considering on ability to produce GABA and also act as probiotics. After that to enhance GABA content in Nham with an acceptable taste, the following factors; MSG, salt and inoculum sizes were optimized using RSM. To produce a safety Nham, the role of starter cultures to reduce BAs and cholesterol were also investigated. Succession of LAB during fermentations of inoculated and uninoculated Nham products was monitored by both the conventional method and the DGGE technique.

Anticipated Outcomes

1. This study we obtained two promising LAB strains (*Pediococcus pentosaceus* HN8 and *Lactobacillus namurensis* NH2) that seemed to have all the right probiotic properties.

2. A general health improvement of Thai fermented pork sausage (Nham) with high amount of GABA, but lower in amounts of BAs and cholesterol to be in safe levels could be achieved by using the starter cultures (*P. pentosaceus* HN8 and *L. namurensis* NH2).

3. In addition of conventional methods, PCR-DGGE with V3 region of 16S rRNA gene was parallel analysis for studying LAB succession in fermentations of inoculated and uninoculated Nham. Overall results demonstrated that both approaches are necessary for providing sufficient data to achieve a complete knowledge of LAB successions in Thai fermented pork sausage (Nham) between inoculation and without inoculation.

CHAPTER 2

LITERATURE REVIEW

1. Fermentation of meat

Meat fermentation, a worldwide and the oldest technique in food preservation, was originally used to store food for longer times (Hutkins, 2006). This principle is not only employed to ensure microbiological safety, but also to enhance the flavor and nutritional quality of product. This technique uses a low energy conservation of meat and remains to be considered as yielding a high quality product. Traditional fermentation relies on natural contamination by environmental microflora. During fermentation, complex biochemical and physical reactions take place resulting in a significant change of sensorial characteristics. Fermentation causes an increase in organic acids along with a concomitant decrease in pH due to the fermentation of added carbohydrates, i.e., sugar. The main fermentation product produced by lactic acid bacteria (LAB), lactic acid, serves to lower the pH and contributes to the stability of these fermented products against food-borne pathogens and other undesirable microorganisms. Besides lactic acid, there are a variety of other end products that are formed during the fermentation process. These include other organic acids, carbon dioxide and alcohols that give distinct flavor and texture of the fermented products. However, fermentation is a biological process; and therefore it is influenced by many factors that need to be controlled in order to produce a safe and consistent product quality.

Problem of biogenic amines in fermented food products

Biogenic amines are organic bases derived from amino acids via the activity of amino acid decarboxylases (Tosukhowong et al., 2011). They are generally classified according to their chemical structures into three categories; aromatic amines (tyramine and phenylethylamine), aliphatic amines (putrescine, cadaverine, spermine

and spermidine), and heterocylic amines (tryptamine and histamine). In addition, they can be classified depending on their synthesis pathways. Natural polyamines (viz. putrescine, spermidine and spermine), referred to as physiological polyamines, are formed during de novo polyamine biosynthesis. These compounds play important roles in the regulation of nucleic acid and protein synthesis, and possibly in the stabilization of membranes. Biogenic amines are of concern for fermented products such as sausages and pickles due to their toxicological effects on nervous, blood pressure, gastric and intestinal systems.

The distribution of the different amines varies according to the food type, with meat being high in spermine, while foods of plant origins contain mostly putrescine and spermidine (Bardocz, 1995). In general, histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermine, and spermidine are considered as being the most important biogenic amines in foods (Shalaby, 1996). In meat, the decarboxylation process can be catalyzed by naturally occurring endogenous decarboxylase or by exogenous enzymes released by various microorganisms associated with the meat products. In the latter situation, the amount of bacterial decarboxylase released is influenced by the nature of the microflora and the composition of the meat product. However, the endogenous production of cadaverine, putrescine and histamine is insignificant when compared with exogenous production (Silla-Santos, 1996). However, the ability of microorganisms to decarboxylate amino acids is highly variable due to the differences in species, strains and environmental conditions (Bover-Cid et al., 2001). The function of biogenic amine production in microorganisms is believed to be a protective mechanism to maintain the intracellular pH homeostasis during growth under acidic conditions (Pereira et al., 2009). Foods that are more likely to contain high levels of these biogenic amines are dairy products, fish and fish products, and meat including meat products like Nham (Suzzi and Gardini, 2003; Tosukhowong et al., 2011).

2. Lactic acid bacteria

Lactic acid bacteria are Gram-positive, acid tolerant and non-spore forming cocci and rods. They are a heterogeneous group of bacteria comprising about 20 genera within the phylum firmicutes (Axelsson, 2004). From a practical point of view the genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* have been considered as the principal LAB (Holzapfel et al., 2001; Axelsson, 2004). Some of these bacteria have been used as starter cultures for promoting meat fermentation (Papamanoli et al., 2003). LAB improve safety and stability of the product (Coppola et al., 1998; Papamanoli et al., 2003); and their long history of safe use commonly referred to as the GRAS (Generally Recognized As Safe) status, combined with a variety of interesting metabolic characteristics have led to a wide range of industrial applications in fermented food products (Holzapfel et al., 2001).

2.1. Taxonomy and physiology

The inability to synthesize porphyrin (e.g., heme) results in the LAB being devoid of catalase and cytochromes (without supplemented heme in the growth media). Therefore, the LAB do not possess an electron transport chain and rely on fermentation to generate energy (Axelsson, 2004). The LAB have two main hexose monosaccharides fermentation pathways, which are homolactic fermentation, in other words glycolysis (Embden- Meyerhof-Parnas pathway, EMP) including the genera *Lactococcus, Streptococcus* and *Pediococcus*; and heterolactic fermentation, via the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway and produce a number of products besides lactic acid, such as carbon dioxide, acetic acid, and ethanol from the fermentation of glucose including the genus *Leuconostoc* and a subgroup of the genus *Lactobacillus*. Based on these two main fermentation pathways the LAB have been divided into two metabolic categories as homofermentative and heterofermentative. However most of LAB are *Lactobacillus* spp. Among lactobacilli are divided into 3 groups (Axelsson, 2004; Felis and Dellaglio, 2011).

2.1.1. Group I, obligate homofermentative lactobacilli, can convert hexose into lactic acid via the EMP (Figure 2.1), but they are unable to ferment pentoses or gluconate due to they lack of phosphoketolase.

2.1.2. Group II, facultative heterofermentative lactobacilli, usually ferment hexoses homo-fermentative into lactic acid, but in some strains and under some conditions, hetero-fermentative metabolism (Figure 2.2) can convert hexose into lactic acid, carbon dioxide and ethanol (or acetic acid). Hence, they have both aldolase and phosphoketolase. Acetic acid production occurs under conditions where NAD^+ can be regenerated without the formation of ethanol. Pentoses are fermented into lactic and acetic acids via a phosphoketolase pathway. The phosphoketolase pathway is repressed in the presence of glucose.

2.1.3. Group III, obligate heterofermentative lactobacilli (Figure 2.2), hexoses are fermented by only phosphoketolase pathway to lactic acid, carbon dioxide and ethanol (or acetic acid in the presence of an alternative electron acceptor). In addition, pentoses are also converted to lactic and acetic acids.

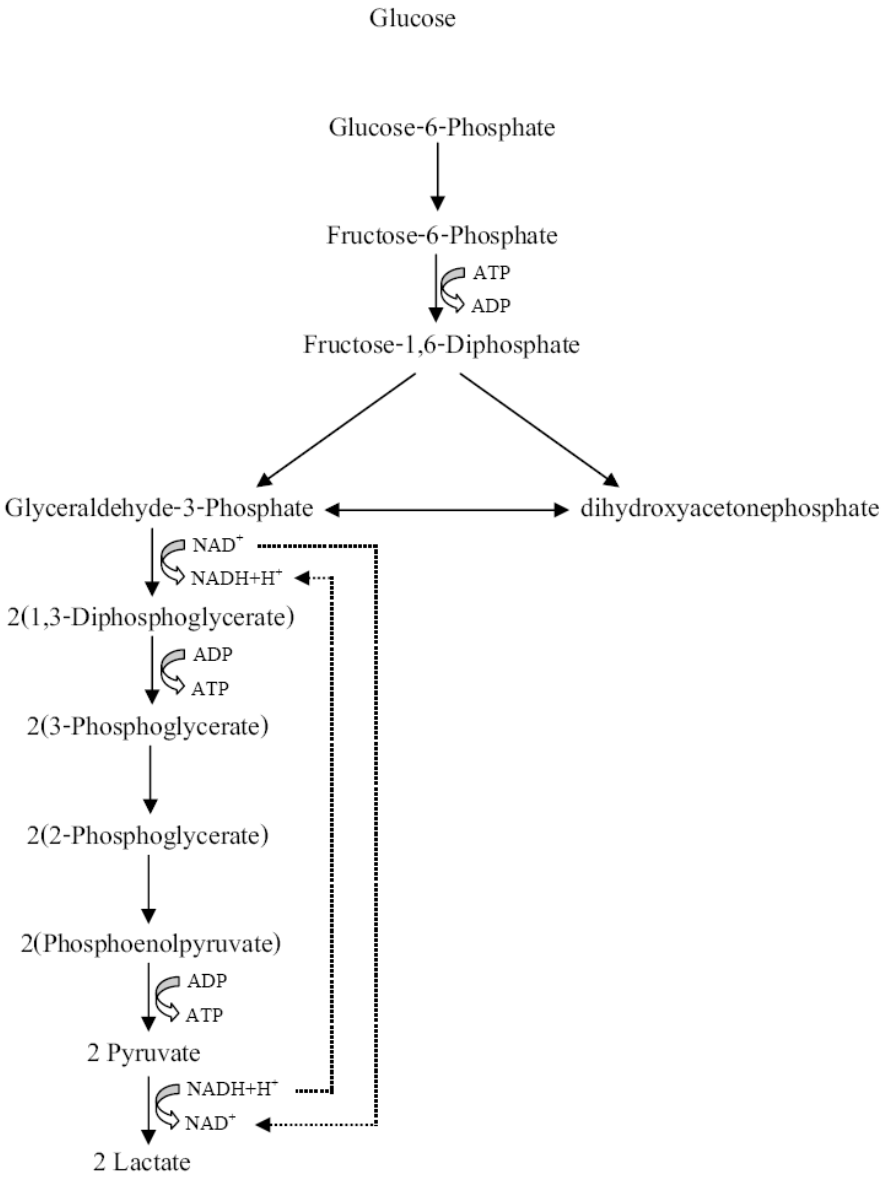


Figure 2.1. The homofermentative pathway (Embden- Meyerhof-Parnas pathway, EMP) of lactic acid bacteria.

Source: Axelsson (2004)

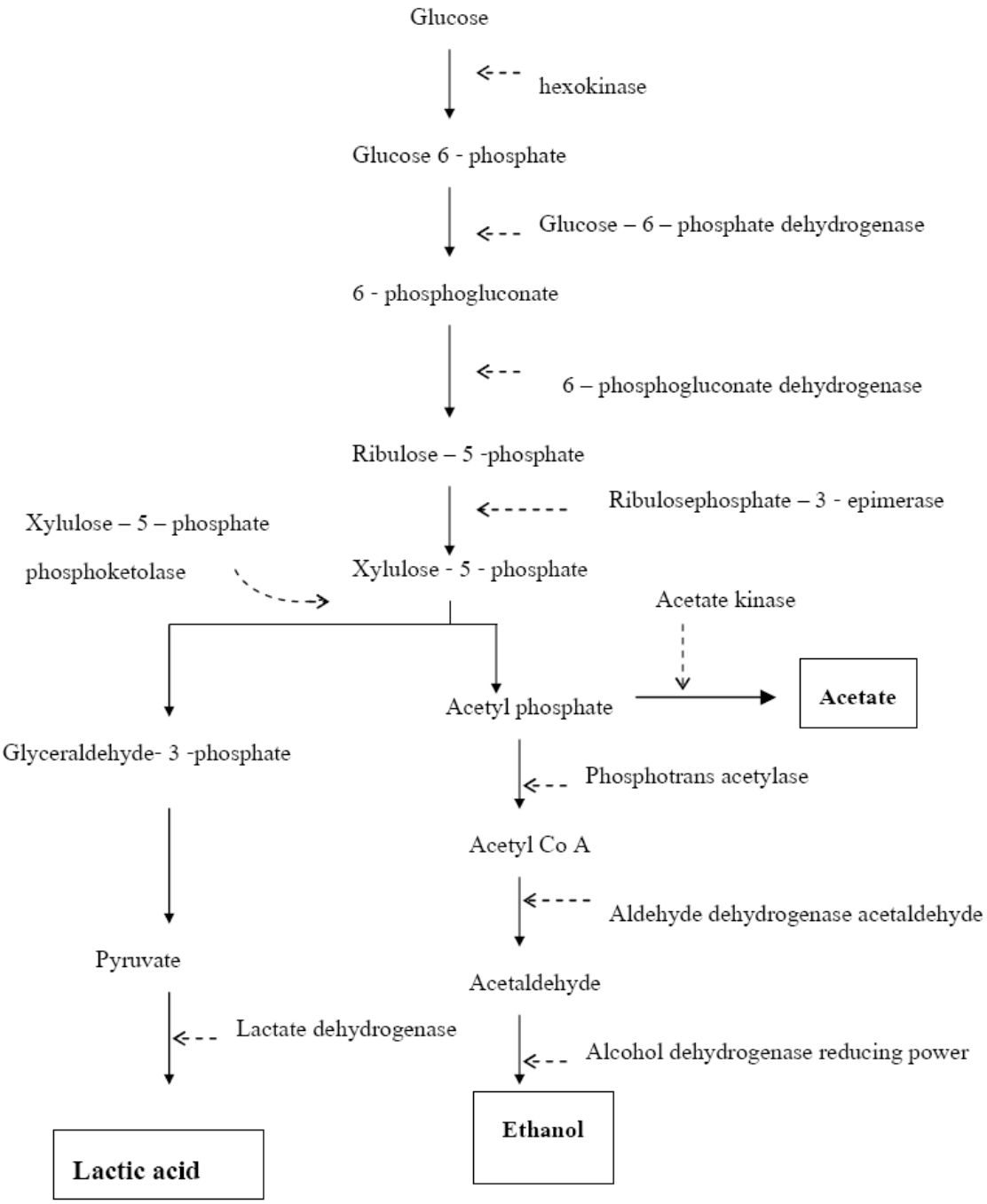


Figure 2.2. The heterofermentative pathway (phosphoketolase pathway) of lactic acid bacteria.

Source: Axelsson (2004) and Aarnikunnas (2006)

In addition, another group of LAB that has important properties in an applied context, especially in the area of food and probiotics namely bifidobacteria, they degrade hexoses through a peculiar metabolic pathway, the so-called bifid shunt, i.e. the fructose-6-phosphate pathway (Figure 2.3), the key enzyme of which is fructose-6-phosphoketolase (Felis and Dellaglio, 2011).

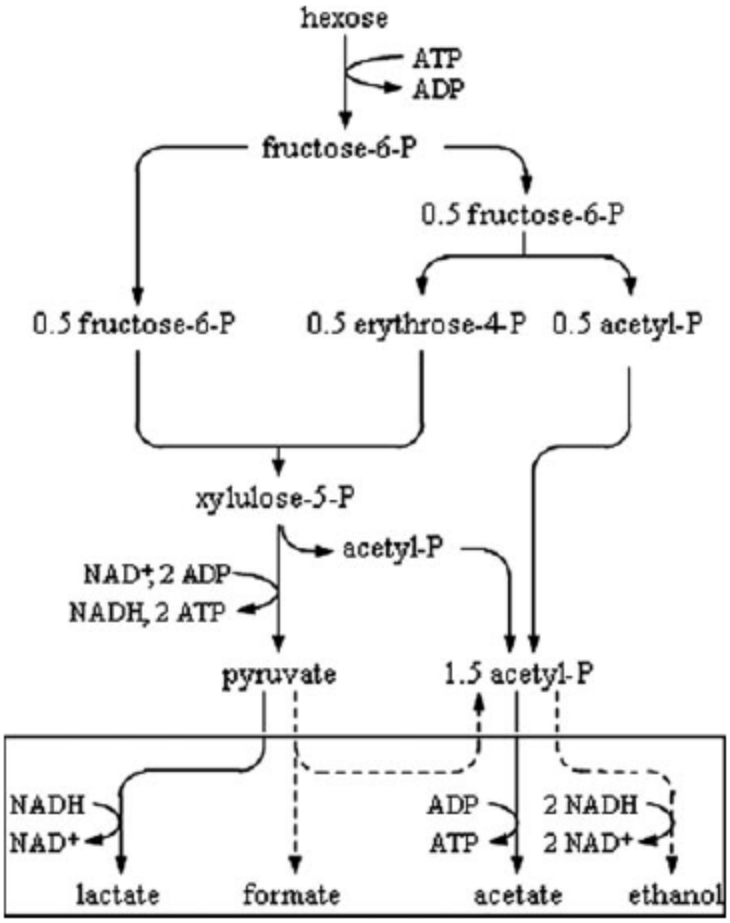


Figure 2.3. The fructose-6-phosphate shunt in Bifidobacteria. Hexose is broken down to 1 pyruvate and 1.5 acetylphosphate, which can give alternative end products (in rectangle). Minor pathways are shown with dashed lines.

Source: Felis and Dellaglio (2011)

2.2. LAB in fermented meat products

In spontaneous meat fermentation, the LAB derived from the raw materials or the environments are responsible for both lactic acid production resulting from carbohydrate utilization and also a low pH value (5.9-4.6). As a consequence of this, the muscle protein coagulated, resulting in the slice ability, firmness and cohesiveness found in the final product. Ripening is also favored when pH values decrease and approach the isoelectric point of proteins. The development of curing color occurs also in acidic condition when nitric oxide is produced from nitrate and can then react with myoglobin (Hugas and Monfort, 1997; Leroy et al., 2006). In meat fermentations, LAB have generally three different purposes; first, to improve safety by inactivating pathogens; second, to improve the product stability and shelf life by inhibiting undesirable changes brought about by spoilage microorganism or antibiotic reactions; third, to provide diversity by the modification of raw material to obtain new sensory properties (Lücke, 2000). The inhibition of pathogenic and spoilage bacteria is a consequence of the accumulation of lactic acid as well as acetic acid, formic acid, ethanol, ammonium, fatty acids, hydrogen peroxide, acetaldehyde, antibiotics and bacteriocins (Hugas and Monfort, 1997).

Nowadays, the consumers pay a lot of attention to the relation between food and health (Leroy and De vuyst, 2004). Therefore, the use of LAB as starter cultures or protective co-culture in the *in situ* control of food pathogens is one of the possible ways to improve food safety. LAB originating from fermented meats is particularly well adapted to the ecology of meat fermentation. The first stage in the starter culture designing process is to characterize LAB isolated from the given meat products, in order to select the best strains (Ammor et al., 2005). Thus, many studies have focused on isolation of LAB that produce antimicrobial substances to inhibit undesirable microorganisms.

2.2.1. Antimicrobial compounds produced by LAB

Organic acids

Lactic acid is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. Acetic and propionic acids are produced by LAB strains through heterofermentative pathway, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang et al., 1986). They are more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of undissociated acids than lactic acid at a given pH (Earnshaw, 1992).

Hydrogen peroxide

Hydrogen peroxide **(**H_2O_2) is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine hydroxyl dinucleotide (NADH) peroxidase (Byczkowski and Gessner, 1988). The antimicrobial 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 membrane lipids thus the increased membrane permeability (Kong and Davison, 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O^{2-}) and hydroxyl (OH^-) radicals which can damage DNA (Byczkowski and Gessner, 1988). It has been reported that the production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* sp. and various psychrotrophic microorganisms in foods (Cords and Dychdala, 1993; Davidson et al., 1983).

Carbon dioxide

Carbon dioxide (CO_2) is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating 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).

Fatty acids

Under certain conditions, some lactobacilli and lactococci possessing lipolytic activities may produce significant amounts of fatty acids, e.g. in dry fermented sausage (Sanz et al., 1988). The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on chain length, concentration, and pH of the medium (Gould, 1991). The antimicrobial action of fatty acids has been thought to be due to the undissociated molecule, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at a lower pH (Kabara, 1993).

Bacteriocins

LAB-bacteriocins are ribosomally synthesized, small, secreted, cationic peptides, which kill target cells by permeabilizing the cytoplasmic membrane (De Vuyst and Leroy, 2007; Gillor et al., 2008). Bacteriocins produced by LAB are classified into three main groups, lantibiotics being the most documented and industrially exploited. The groups are lantibiotics (Class I), nonlantibiotics, small heat-stable peptides (Class II) and large heat-labile protein (Class III) (Drider et al., 2006). To date, nisin A is the only bacteriocin that has been approved for commercial use in many countries; and it is the best suited for use in acidic foods (Delves-Broughton, 2005). One drawback of the direct application of bacteriocins to a food

matrix is that activity loss occurs over time because of enzymatic degradation and interactions with food components such as proteins and lipids (Stergiou et al., 2006).

2.2.2. Probiotics

Probiotics are described as 'live microorganisms which when administered in adequate numbers confer a health benefit on the host' (FAO/WHO 2001, 2002; Bhadoria and Mahapatra, 2011). In the last decade there has been an increasing in using LAB as probiotics because several beneficial functions have been reported for probiotic bacteria, e.g. vitamin production, production of important digestive enzymes, prevention and treatment of diarrhea, establishment of a healthy flora in premature babies, alleviation of the symptoms of lactose maldigestion, stimulation of the immune system, suppression of tumorigenesis, and cholesterol reduction (O'Sullivan, 2001; Holzapfel and Schillinger, 2002; Zhang et al., 2007). The important criteria for a LAB strain to be probiotic are, in addition to acid and bile tolerance, the ability to produce antimicrobial compounds against pathogenic bacteria, and to adhere for colonization on human intestinal mucosa (Khan and Ansari, 2007). Currently available LAB used as probiotics for human consumption are *Lactobacillus delbreuckii* subsp. *bulgaricus, L. acidophilus, L. fermentum, L. rhamnosus, L. paracasei, L. casei, L. reuteri, L. johnsonii, L. plantarum, L. salivarius, Lactococcus lactis, Bifidobacterium infantis, B. breve, B. longum, B. lactis, Streptococcus salivarius* subsp. *thermophilus, Enterococcus faecalis* and *E. Faecium* (Sanders, 2008). Although the minimum recommended level of viable probiotics present in foods for any health benefits to be achieved can show a discrepancy, in general the food industry has adopted the recommended level of 10^6 CFU (Colony Forming Units)/mL at the time of consumption (Boylston et al., 2004). This standard appears to have been introduced to achieve bacterial populations that are technologically attainable and cost effective (Boylston et al., 2004). United States Food and Drug Administration (US FDA) has also recommended that the minimum probiotic count in a probiotic food should be at least 10^6 CFU/mL. Depending on the

amount ingested and taking into account the best-before date, a regular - in most cases daily intake of 10^8 to 10^9 probiotic microorganisms is necessary to achieve probiotic action in the human organism. The main targets of probiotic intervention have been proposed as given below (Bhadoria and Mahapatra, 2011):

- Increasing natural resistance to infectious disease in the gastrointestinal tract and a first line of defense against disease;

- Prevention of dangerous fungal overgrowth and some allergic reactions;

- Reducing putrefactive/toxic microbial metabolism in the gut;

- Promoting optimized digestive processes, allowing maximum nutritional benefit from food;

- Improved resistance to toxic bowel problems and diarrhea;

- Stimulation of the immune system;

- Production of needed nutrients, like vitamin K, one form of which the body itself cannot make;

- Improving lactose intolerance conditions;

- Reduction of cholesterol levels;

- Act as antioxidants;

- Controlling diseases where components of the intestinal microbiota have been implicated in aetiology.

Many scientists have recently proposed the use of probiotic meat starter cultures for dry fermented sausage manufacture (Hammes and Knauf, 1994; Pennacchia, et al., 2004; Ruiz-Moyano et al., 2009, 2011a, b). In addition, Ruiz-Moyano et al., (2011a, b) reported that the potentially probiotic strains *L.reuteri* PL519, *L. fermentum* HL57 and *P. acidilactici* SP979 are applicable for enhancement safety of Iberian dry-fermented sausages.

Probiotic properties

The criteria used to select potential probiotics are related to acid and bile tolerance, production of antimicrobial substances, cholesterol removal, production of useful enzymes and safety for food and clinical use. *In vitro* survival of bacterial strains in low pH is a more accurate indication of the ability of strains to survive passage through the stomach. The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 for 2-4 h depending on the individuals, diet and the prevailing conditions (Pennacchia et al., 2004).

Bile salt tolerance is the second selection criterion for probiotics. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to 700 mL per day. The relevant physiological concentrations of human bile range from 0.1 to 0.5% (Mathara et al., 2008). Hence, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v). Bile salt hydrolytic (BSH) activity may contribute to resistance of LAB to the toxicity of conjugated bile salts in the duodenum and therefore is an important colonization factor. The deconjugation activity may play a role in maintaining the equilibrium of the gut microflora. It has been also suggested that bile BSH enzyme might be a detergent shock protein that enables LAB to survive the intestinal bile stress. Cell hydrophobicity is one of factors that may contribute to adhesion of bacterial cells with the host tissue. *In vitro* test, the highest value of 36.7% hydrophobicity was found by toluene and 23.1% in hexadecane (Ram and Chander, 2003).

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, the antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material (Mathur and

Singh, 2005). One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable resistance genes. Safety is one of the recommended attributes in the FAO/ WHO (2002) guidelines on evaluation for probiotics. Haemolysis activity would break down the epithelial layer while gelatinase activity would derange the mucoid lining. These impairments interfere with the normal functioning of these very important linings and would cause pathways for infections. Absence of haemolytic and gelatinase activity is a selection criteria for probiotic strains, indicating that these bacteria are none virulent (De Vuyst et al., 2003).

Safety and technological aspects of probiotics

The safety of probiotics can be described as centuries of use fermented products; no reports of probiotic pathogens and safe use of active cultures in thousands of subjects have demonstrated that probiotic intake is safe. This past safe history is very important regarding use by pregnant woman and newborn, because there is some limitation for clinical trials. At the same time, some scientists have doubt about reasonability in taking a high dose of viable bacteria (Henriksson et al., 2005). A review outlining the safety of current probiotic compounds has been published (Borriello et al., 2003). Cases of infection caused by lactobacilli and bifidobacteria are extremely rare. Indeed, the concept of a balance existing in the intestine, involving competition between probiotics and pathogenic bacteria for specific binding sites on intestinal epithelial cells has been well established in the literature. However, recent research has turned toward understanding the role of probiotics and their products in enhancing and modulating innate, and adaptive immune responses in the organism by other mechanisms (Fedorak and Madsen, 2004).

Functional foods with probiotics are now well established on the European market. Starting about 20 years ago these products range have increased (Saarela

et al., 2000) and is presently known to most consumers. To succeed in promoting the consumption of functional probiotic products the food industry has to satisfy the demands of the consumers. Foods used for dissemination of probiotics are usually fermented foods even if probiotics also could be present in infant formula, fruit drinks, whey drinks and sweet milk. Fermented milk and cheese are the most common foods with probiotics (Svensson, 1999).

Healthful role of probiotics

Cholesterol removal

It is thought that the mechanisms by which LAB exert their effect on cholesterol is through bile acids. The liver uses cholesterol to produce bile acids, which are secreted into the small intestine, and then absorbed again and sent back to the liver. Whilst these bile acids are in the intestine however they can be broken down by certain kinds of bacteria that inhabit the gut (Hentges, 1983). Several studies indicated that *Lactobacillus* species are able to reduce cholesterol via several mechanisms including bile salt deconjugation (Liong and Shah, 2005). Other hypocholesterolemic mechanism(s) of LAB may be involved in the removal of cholesterol from the growth media. The removal of cholesterol by LAB *in vitro* could be due to an uptake or assimilation of cholesterol by bacterial strains. Liong and Shah (2005) demonstrated that a portion of the cholesterol assimilated by *Lactobacillus* strains was incorporated into the cellular membrane. Sirilun et al., (2010) demonstrated that the four *L. plantarum* isolated from food origins were considered as the effective probiotics with cholesterol-lowering property capable of reducing 25.41% to 81.46% from the growth medium after 24 h of incubation. Sieladie et al., (2011) reported that 15 tested strains of *L. plantarum* removed 56.52 to 95.65% cholesterol from the growth medium after 18 h of incubation and demonstrated cholesterol assimilation capacity of 16.20 to 34.41 mg of cholesterol per g of cells.

Fifty four volunteers participated in a randomized cross over trial; the results of which revealed reductions of between 5-10% in serum cholesterol levels after several weeks of moderate consumption of yoghurt fermented with *L. bulgaricus* and *S. thermophilus* (Guarner and Schaafsma, 1998).

2.2.3. γ-aminobutyric acid (GABA)

GABA is a non-protein amino acid compound that is synthesized by decarboxylation of glutamic acid via the glutamate decarboxylase enzyme (Komatsuzaki et al., 2008). Therefore, adding MSG into the LAB culture media can increase the amount of GABA in culture broths (Lu et al., 2008). It is well recognized that GABA has a variety of well-characterized physiological functions i.e. reducing hypertension, diuretic effects, inhibiting proliferation of cancer cells (Park and Oh, 2007) and preventing diabetes (Adeghate and Ponery, 2002). Glutamate decarboxylase (GAD, EC 4.1.1.15) is the key enzyme in the conversion of L-MSG to GABA, which catalyzes the irreversible α-decarboxylation of L-glutamate or glutamine to GABA (Hiraga et al., 2008). When L-glutamate or glutamine enters the cell via a specific amino acid transport apparatus, it may consume protons in the cytoplasm, which causes an increase in internal pH. The GABA produced is exported from the cell via an antiporter. Elevated intracellular and extracellular pH help maintain pH homeostasis when the external pH drops (Figure 2.4). This is the GAD–GABA antiporter system (Richard and Foster, 2004). The GAD–GABA antiporter system may protect cells against acid stress in *E. coli* (Richard and Foster, 2004), *Listeria monocytogenes* (Cotteret et al., 2001), and *Lactococcus lactis* ssp. *cremoris* (Nomura et al., 1999).

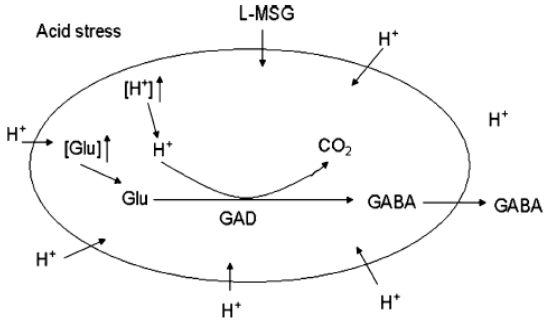


Figure 2.4. Maintenance of pH homeostasis in *L. brevis* NCL912 by GAD–GABA antiporter system. According to this model, acid stress might increase cytosolic H^+ and L-MSG could enter the cell via a specific amino acid transport apparatus, which increases the cytosolic glutamate levels. GAD occurs in the cytoplasm, which consumed H^+, and GABA is exported from the cell via the antiporter. This helps maintain a neutral cytoplasmic pH when the external pH drops. Meanwhile, the increased extracellular GABA levels increase the media pH, which maintains cell pH homeostasis. Glu; Glutamate (Huang et al., 2011)

GABA supplements may not adequately penetrate the blood-brain barrier. The direct addition of chemical GABA to food is considered unnatural and unsafe. So it is necessary to find a natural method to produce and increase GABA in food (Cao et al., 2009). Various types of LAB that show GABA-producing ability is an interesting target for food industry, especially fermented foods because any individual strain has specific fermentation profiles such as acid production, taste and flavor formation ability. These profiles are considered as important factors in the use of LAB as starter cultures in the production of fermented foods. Recent research was undertaken to increase GABA-producing LAB in foods (Huang et al., 2007; Kim et al., 2009; Sun et al., 2009). For instance, *Lactobacillus brevis, L. plantarum, L. paracasei, Streptococcus salivarius* subsp. *thermophilus* Y2, *Enterococcus avium* G-15 and *Lactococcus lactis* are GABA producing LAB (Yokoyama et al., 2002; Li et al., 2008; Lu et al., 2009; Di Cagno et al., 2010; Tamura et al., 2010; Ratanaburee et al., 2011a). Oral administration of GABA in rice germ of 26.4 mg daily has been shown to be effective in treating neurological disorders (Okada et al., 2000) while oral administration of GABA of 10 mg daily for 12 week was effective for hypertensive

patients (Inoue et al., 2003). To our knowledge, no reports have considered fermented sausages as a potential vehicle for GABA.

MSG is used as a substrate for the GABA production by LAB. MSG is the sodium salt of glutamic acid. Glutamate is a naturally occurring amino acid that is found in nearly all foods, especially high protein foods such as dairy products, meat, fish and in many vegetables. Foods often used for their flavoring properties, such as mushrooms and tomatoes, have high levels of naturally occurring glutamate. Glutamate is ubiquitous in nature and is present in all living organisms. It is the principal excitatory neurotransmitter in central nervous system. Glutamate is being used as food additive for enhancing flavor for over last 1200 years imparting a unique taste known as “umami” in Japanese (Beauchamp, 2009). It is being marketed for about last 100 years. The taste of umami is now recognized as the fifth basic taste. The Joint FAO/WHO Expert Committee on Food Additives, based on enumerable scientific evidence, has declared that, “glutamate as an additive in food” so it is not an health hazard to human being. Excessive accumulation of glutamate in the synaptic cleft has been associated with excitotoxicty and glutamate is implicated in number of neurological disorders. As MSG is neurotoxic for less than 5 years old children, so, using an alternative to MSG may be a better substrate for GABA production. Hence, it is very crucial to minimize the usage of glutamate in food industry for human consumption (Farrah et al., 2009; Bamnia, 2011).

3. Thai fermented sausage

3.1. Nham

Nham is a traditional Thai fermented pork sausage popularly consumed in all parts of Thailand. Nham is considered a local authentic due to its flavor and texture varied in accordance which the region in which it is produced (Valyasevi and Rolle,

2002). The main ingredients used in Thai fermented sausages are meat (pork or chicken), salt, nitrite or nitrate, sugar or cooked rice and spices. The mixtures are mixed well and stuffed into casing. Natural fermentation or spontaneous fermentation is at ambient temperature (about 30°C) for 3 to 4 days by predominant bacteria, LAB, until becoming sour and it may be cooked before eating (Thai Industrial Standards Institute, 1994). Currently, the scale of production of Nham has been increasing from household to a small industrial scale with an estimated production value of 20 million USD annually (Visessanguan et al., 2004).

The fermentable carbohydrates are used as carbon source for the sausage microflora to increase acidity of meat by producing organic acids, mainly lactic acid (Leroy and De Vuyst, 2004). The pH drop caused by organic acids production prevents the growth of spoilage and pathogenic bacteria such as *Salmonella, Listeria monocytogenes*, *S. aureus*, and *E. coli* O157:H7. Recommendation on microbiological guideline for Nham by the Thai Industrial Standard number 1219-2547 is no detection of any potential serious pathogen; *Salmonella*, *S. aureus* and *Clostridium perfringens* while fungi must be less than 10 colonies/gram of sample. Whilst the Thai Community Product Standard number 145/2546 also recommends as above but it has one more organism, *E. coli* must be less than 3 MPN/g of sample. However, some contamination of Nham product by *Bacillus cereus* may be possible as this pathogen is normally found in cooked rice as one of the ingredients of Nham. *Shigella sonnei* continues to be a major foodborne threat to public health as it is commonly found in meat (Schimmer et al., 2008) and thus it should be possible to detect in Nham.

Sodium chloride (NaCl) added in the raw sausage mixtures affects microorganism growth, interacts with the myofibrillar, solubilizes proteins, and contributes obviously to the taste of meat products (Yada, 2004). The general consensus of addition of nitrate or nitrite salt to meats is a positive contribution to the color, flavor and shelf life of the product. The curing step has been employed in traditionally made fermented sausages to increase the number of lactic acid, color and flavor forming bacteria (Olesen et al., 2004; Rotsatchakul et al., 2009).

Nitrite and nitrate salts also inhibit the growth of unwanted microorganisms, particularly *Clostridium botulinum* spores which can create a lethal toxin. Nitrite and nitrate salts should be added at a maximum of 125 ppm (parts per million) and 500 ppm, respectively (Retrived January 20, 2012, from http://elib.fda.moph.go.th/). Spices, such as pepper and garlic, have an impact on flavor and they may also have antioxidative and antimicrobial effect (Hammes and Knauf, 1994). On the other hand, sausage production depends on natural fermentation; therefore, product quality varies from batch to batch. Unfortunately, if the normal beneficial microflora does not multiply as usual, the product may be spoiled. It can cause illness due to pathogenic microorganisms or their toxins, and even become lethal due to botulinum toxin production (Woodburn, 1992). It is considered a dangerous process due to the lack of fermentation control. To prevent these problems, the use of starter cultures has become worldwide application in food fermentation

3.1.1. Nham Production

Nham is prepared by combining of minced pork, table salt (NaCl) and sodium nitrite in the first step. The mixture is, then thoroughly mixed with sucrose, monosodium glutamate, erythrobate, phosphate and minced cooked rice and garlic (Figure 2.5). After, thoroughly mixed the mixture was added with shredded cooked pork rinds and chili. Finally, the mixture is stuffed in casings and fermented for 3-5 days at room temperature (~30^{O}C). According to Nham standard TIS 1219-2547 (2004) issued by the Thai Industrial Standards Institute, Ministry of Industry, Nham is recommended to be fermented until pH $\leq$ 4.6 before consumption (Chokesajjawatee et al., 2009). During fermentation, complex changes in microbiological, physicochemical and biochemical characteristics take place that result in a significant change of Nham characteristic (Visessanguan et al., 2006a). Mechanical processes for the production of Nham are well developed; but the fermentation process is remained rely on contamination of desire microorganisms. Acid production contributes to both intrinsic quality and safety in the production of Nham, including sour taste, typical fermented aroma, firmness of texture and as well as prevent the growth of acid

sensitive pathogens (Valyasevi and Rolle, 2002; Visessanguan et al., 2004). At the end of fermentation, Nham usually has a pH of 4.4–4.8 with titratable acidity values of 0.77–1.60% (Phithakpol et al., 1995). Methodologies for the production of Nham are summarized in Figure 2.5.

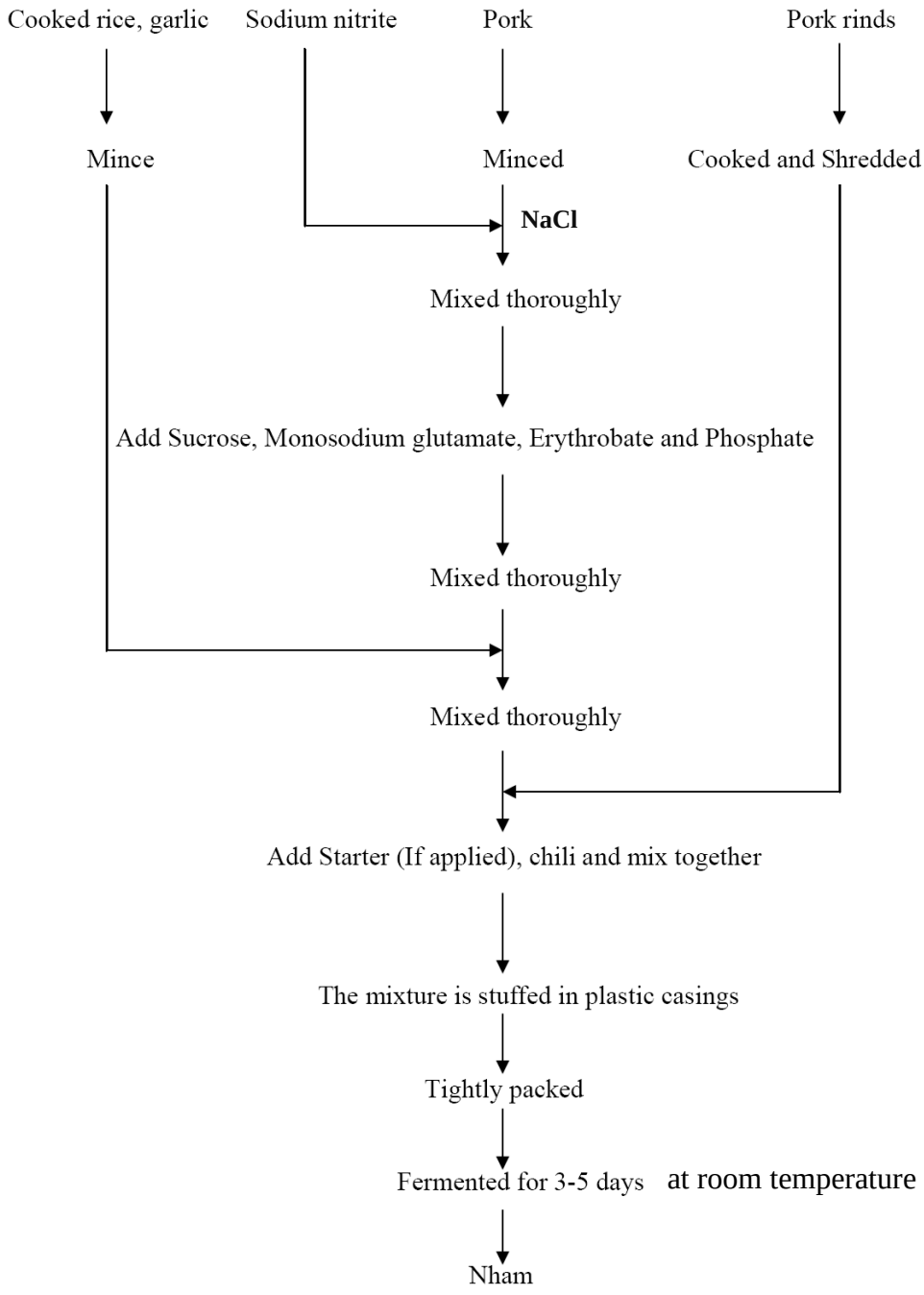


Figure 2.5. The flow chart of the manufacturing process of Nham

Source: Modified from Phoonsawat (2005) and Kongtong et al., (2011)

3.1.2. Ingredients of Nham

Ingredients of Nham, according to Visessanguan et al., (2004) are listed below;

Formula for 1 kg of Nham

Ingredients	Weight (g)
Minced pork	520
Cooked pork rind	350
Garlic	43
Cooked rice	43
Whole bird chili	20
Salt	19
Sucrose	4
Monosodium glutamate	2
Erythrobate	2
Trisodium polyphosphate	2
Potassium nitrite	0.1

3.1.3. Functional properties of ingredients during Nham fermentation

The success in production of Nham is influenced by several factors, including natural microflora, quantity and properties of raw ingredients (e.g. meat, salt, sucrose, erythrobate, phosphate, nitrate and nitrite). The varying proportion of these

ingredients is thought to play important roles in chemical, physical and sensory properties of Nham.

3.1.3.1. Pork and Pork rind

Minced pork and cooked pork rind are two major typical ingredients used in Nham, comprised over 90% of the ingredients. Pork meat should be fresh in order to minimized bacterial contamination (Kunawasen, 2000). Proteins, derived from raw meat and cooked pork rind, exhibit a wide range of functional properties. They are able to form networks structures, interact with others ingredients, and thus play an important role in the textural, sensory, and nutritional quality of foods. Visessanguan et al., (2005) reported that the proportion of minced pork and rind in the formulation could affect texture, color, water binding capacity, and sensory quality, but not the fermentation characteristic of Nham ($p>0.05$). However, the ratio of minced pork to rind at 1:1 was the most appropriate to minimizing the cost of production for the Nham formulation.

3.1.3.2. Cooked rice

Cooked rice can be used as the carbon source by LAB. Wiriyacharee et al., (1994) found that Nham formulation consisted of 3% cooked ordinary rice and 1% cooked glutinous rice, exhibited low pH (pH 4.3) and high total acid (1.1%) within 48 hour at 30ºC. Cooked rice is the suitable source for Nham production which using starter cultures (*L. plantarum, P. acidilactici* and *M. varians*) (Opaswatcharanon, 2004).

3.1.3.3. Garlic

Garlic has an impact on flavor and it may also have antioxidant and antimicrobial effects (Tyopponen et al., 2003). Swetwiwathana et al., (1999) found that garlic enhanced the growth of three commercial meat starter cultures (*L. curvatus*, *L. sake* and *P. acidilactici*) for increasing the lactic acid production and rapid decreasing of pH value, which leaded to shorten Nham fermentation time.

3.1.3.4. Nitrate and nitrite ions

Nitrate and nitrite ions are curing reagents which play an important role in the color development as well as can inhibit pathogen growth. Nitrate is reduced to nitrite by bacteria or acid condition. In the first step of color development in meat product, nitrite is reduced to nitric oxide in acidic condition. The occurring nitric oxide reacts with myoglobin to form nitrosomyglobin which exhibit red color (Opaswatcharanon, 2004). The mechanism of color development is shown in Figure 2.6 Nitrite in the form of undissociated nitrous acid (HNO_2) is able to pass the ion barrier of bacterial cell wall and disturb the function of bacterial enzyme and resulting to inhibit bacterial growth (Tyopponen et al., 2003). Wiriyacharee et al., (1995a) reported that *Kocuria varians*, formerly known as *Micrococcus varians* is important in converting nitrate to nitroso-haemoglobin (NOMb), thus, imparting a pink color to Nham. The maximum allowable level of nitrite residue is 125 ppm (Codex, 1994). Since the excess of residual nitrite can react with secondary amines in certain meat products to form nitrosamine which are proved to be carcinogen (Kunawasen, 2000). Therefore, it is recommended to use an effective minimum concentration in producing Nham.

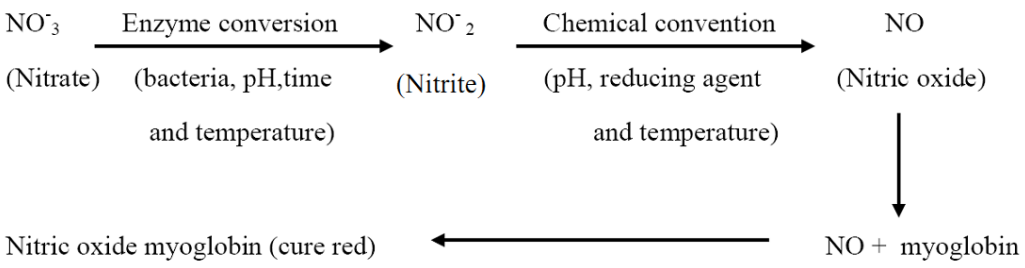


Figure 2.6. Simplified mechanism of curing agent for color development in sausage.

Source: Modified from Opaswatcharanon (2004)

3.1.3.5. Sugar

Sucrose (table sugar) or glucose is added as fermentable substrates for growth of LAB, and production of acid (Tyopponen et al., 2003). Wiriyacharee et al., (1995) found that Nham formulation consisted of 5% glucose, exhibited low pH (pH 4.2) and high total acid (1.0%) within 48 hour at 30˚C. The obtained products have a good sensory quality.

3.1.3.6. Salt

Table salt (NaCl) is an important ingredient to enhance the organoleptic properties of Nham and to inhibit the growth of undesirable microorganism (Kunawasen, 2000). The amount of salt that used in Nham inhibits the growth of spoilage bacteria contaminated in pork; but it does not inhibit growth of fermentative bacteria. It also induces the solubility and diffusion of myofibrillar proteins from muscle forming gel between meat and fat particles of the raw sausage material (Tyopponen et al., 2003). Generally, the amount of salt in fermented sausage

is in the range of 2.5-3.5% (Opaswatcharanon, 2004). Techapinyawat (1975) found that Nham containing 3% salt produced the highest score from sensory analysis. Moreover, salt concentration lower than or equal to 2% can cause fermentation failure while salt concentration higher than 4% can increase fermentation time and too salty.

3.1.3.7. Erythrobate and ascorbic acid

Erythrobate and ascorbic acid compounds are used in meat products to improve color of products by enhancing the color formation. They act as catalyst in development and maintenance of pink-red color in meat. There were several reports which showed the effect of using the combination of ascorbic acid and nitrite compound to improve color formation in pork product (Thungtakul, 1988; Dandamrongrak, 1991). The color, flavor and taste of pork product were improved when used both ascorbic acid and nitrite compound (Opaswatcharanon, 2004).

3.1.3.8. Phosphate compounds

Phosphate compounds are widely used in meat products. These compounds improve the chemical and physical properties of the product such as texture, color, stability and water absorption (Rotsachakul, 2002; Munikanonth, 2004). In addition, phosphate compounds also have buffering capacity which can prevent an increase in pH during early period of meat fermentation, and can prolong the time before pH dropping (Opaswatcharanon, 2004).

3.2. Microbiological changes during Nham fermentation

In spontaneous fermentation, the raw materials are converted by natural microflora to products that have acceptable food qualities. Therefore, the quality of the product is depends on the occurrence of desirable microorganisms in the ingredients used. Over the past decades, the scientific community has paid special attention to the correct identification of microorganisms involved in Nham fermentation as well as the role of microorganisms during fermentation to find potential starter for ensuring safety and stability of Nham products. Several reports have focus on the isolation and identification of microflora from commercial Nham samples during fermentation. In previous studies, pediococci (*Pediococcus* spp., *P. cerevisiae* (reclassified as *P. pentosaceus*) and heterofermentative lactobacilli were found to be dominated at the early stage of fermentation and lactobacilli (*Lactobacillus* spp., *L. plantarum* and *L. brevis*) grew at the later stage and became dominance at the end of fermentation (Techapinyawat, 1975). Several studies on Nham microflora have shown lactobacilli (*L. plantarum*, *L. pentosus* and *L. sakei)* and pediococci (*P. acidilactici* and *P. pentosaceus*) are the dominant microorganisms in Nham fermentation (Tanasupawat and Komagata, 1995; Valyasevi and Rolle, 2002). Other microorganisms that found at early phases of the fermentation include *Micrococcus varians*, yeasts and molds. Khieokhachee et al., (1997) reported a total non-lactic acid bacterial count in the order of 10^6 CFU/g, and yeasts count in the order of 10^3 CFU/g during the initial 16 h of Nham fermentation. Recently, Kongtong (2011) focused on the identification of LAB in Nham from different commercial brands by using both phenotypic and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to confirm the identification of LAB strains during Nham fermentation. The results from this study showed that the dominant genetic groups are members of lactobacilli including *L. garvieae, L. plantarum, Lc. lactis, P. pentosaceus* and *P. acidilactici.*

Nham fermentation is usually resulting in product of inconsistent quality due to difficulty in control and/ or prediction of the process as well as increasing risk of

pathogenic bacterial growth. Somathiti (1982) examined coliform counts, *Salmonella* spp. and *Shigella* spp. in commercial Nham samples from 4 provinces of Thailand. This study found that the average of coliform counts were 1.1-6.1 x 10^4 CFU/g, 2.5 x 10^5 CFU/g, 2.5 x 10^5 CFU/g and 30 CFU/g, in Nham products obtained from Bangkok, Chiang Mai, Chiang Rai and Ubonratchathani, respectively. In addition, *Salmonella* spp. was detected at 12%, 25%, 42% and 11% in Nham obtained from Bangkok, Chiang Mai, Chiang Rai and Ubonratchathani, respectively. However, none of these samples were found to contaminate with *Shigella* spp. Paukatong and Kunawasen (2001) and Chokesajjawatee et al., (2009) reported that food-borne pathogenic bacteria such as *Salmonella* spp., *S. aureus* and *L. monocytogenes* can be found in Nham with pH higher than 4.6 at 16%, 15% and 12%, of commercial Nham samples, respectively. This renders the product to be unsafe for consumers. Therefore, the safety of Nham fermentation relies on the rapid growth of LAB and a rapid decrease of pH value to minimize the risk of the growth of food-borne pathogens.

The complexity and variability of microbial populations associated with spontaneous fermentation can partially reduce in back-slopping practice, i.e. inoculation of the raw material with a small quantity of a previously performed successful fermentation (Holzapfel, 2002; Leroy and De Vuyst, 2004). Techapinyawat (1975) found that Nham fermentation which was operated by back-slopping, exhibited a higher rate of fermentation than naturally fermented Nham as indicated by greater rate of pH drop and acidity production. Based on pH and total acidity, the fermentation completed within 96 and 36 h for control and back-slopping, respectively. This might indicated the potential of using starter cultures in Nham fermentation. Currently the use of starters as functional flora is gaining importance. Starter cultures are widely used for producing various kinds of fermented meat products including Nham to shorten the fermentation time, ensure proper acid production and the color development, enhance the flavor and improve the product safety, and inhibit undesirable microorganisms.

3.3. Improvement of sausage products using starter cultures

A meat starter cultures can be described as viable microorganisms added directly to meat in order to improve the storage quality, safety and/or enhance consumer acceptability of meat product (Kunawasen, 2000). A wide variety of microorganisms, mainly LAB, *Staphylococcus* sp. and *Kocuria* spp., have already been isolated from sausage fermentations. These microorganisms have been selected for using in meat ecosystems, to improve the quality and safety of the final fermented meat products (Baruzzi et al., 2006).

The most important microorganisms used in starter cultures are presented in Table 2.1. The advantages of starter cultures are complete fermentation, control of fermentation rate, reduction of fermentation time, reduction of formation of off flavors, formation of more consistent flavor characteristics and improvement of quality and profitability. Starter cultures also provide safety by competing for food with undesirable bacteria thus inhibit their growth (Hugas and Monfort, 1997).

Table 2.1. The important microorganisms used in starter cultures for sausage production (Sriphochanart, 2009)

Microorganisms	**Species**	**Characteristics**	**References**
LAB	*Lactobacillus plantarum*	acid production	Hugas et al., 1993
	L. pentosus		Rebecchi et al., 1998
	L. sakei		Papmanoli et al., 2003
	L. curvatus		Visessanguan et al., 2006
	Pediococcus acidilactici	acid production (fast fermenting)	Vural, 1998
	P. pentosaceus		Rebecchi et al., 1998
Curing bacteria	*Kocuria varians*	color and flavor	Arihara et al., 1993
(color and flavor	*(Micrococcus)*		
forming)	*Staphylococcus xylosus*	color and flavor	Talon et al., 2002; Olesen et al., 2004
	S. carnosus		Stahnke et al., 2002
	S. equorum		Mauriello et al., 2004
Yeasts	*Debaryomeces hansenii*	flavor	Durá et al., 2004
	Candida famata	flavor	Olesen and Stahnke, 2000
Molds	*Penicillium nalgiovense*	color and flavor	López-Díaz et al., 2001
	P. chrysogenum		Sunesen and Stahnke, 2003

Starter cultures can be implemented for varieties of purpose as described below.

3.3.1. Starter cultures for a better taste of product

Basic flavor results from the interaction of taste (mainly determined by lactic acid production and the pattern of peptides and free amino acids resulting from tissue-generated proteolysis) and aroma (mainly determined by volatile components derived from bacterial metabolism and lipid autoxidation) (Leroy et al., 2006). Lactic and acetic acids are often suggested to be major contributors to the acid aroma and taste and the development of the Nham's texture (Visessanguan et al., 2004). The autooxidative phenomenon, especially lipid oxidation in Nham, was likely to be intense and might partially contribute to the Nham characteristics (Visessanguan et al., 2006). The LAB neither possessed high proteolytic nor lipolytic capabilities. Since microbial lipases generally are very sensitive to pH, lipolysis at conditions relevant to Nham fermentation was likely mediated by lipases present in lysosomes of the muscle tissues (Visessanguan et al., 2006). Various *Staphylococcus* spp. are often used as starter cultures in production of dry fermented sausages due to their nitrate reductase activity and flavor enhancing capacity. Additionally, *Staphylococcus* often possesses catalase activity which indirectly could alter flavor formation by preventing chemical oxidation of fatty acids (Talon et al., 1999; Tjener et al., 2003). Staphylococci, in particular *Staphylococcus xylosus* and *S. carnosus*, modulate the aroma through the conversion of amino acids (particularly the branched chain amino acids; leucine, isoleucine, and valine) and free fatty acids. In addition, additives such as nitrate, nitrite, or ascorbate, pre-cultivation parameters and environmental factors clearly influence the generation of aroma compounds (Leroy et al., 2006).

3.3.2. Starter cultures for a safe product

The use of functional starter cultures may be useful to reduce levels of nitrite and nitrate, which received a lot of attention because of their contribution to the formation of health affecting nitrosamines (Leroy et al., 2006). The primary species of bacteria successfully used as starter cultures are *Lactobacillus* sp., *Pediococcus* sp., and *Micrococcus*/*Staphylococus* sp. Lactic acid is produced from carbohydrates by genera of *Lactobacillus* sp., *Pediococcus* sp., which leads to a drop in pH and inhibits the growth of undesirable microorganisms in the products. Moreover, contributes to the development of flavor. *Micrococcus* sp. and *Staphylococus* sp. reduce nitrate and ensure the color development (Swetwiwathana et al., 1999). In contrast, Chiwprasertphol (2002) showed that *Lc. lactis* BCC 11499 could compete with microflora in Nham at the early stage of fermentation. Moreover, low concentration of sodium chloride and nitrite as using in Nham does not effect on growth and bacteriocin production of *Lc. lactis* strain BCC 11499. Therefore, the application of *Lc. lactis* strain BCC 11499 as a starter culture in Nham fermentation has been developed. These observations suggest that BCC 11499 strain may be used as co-cultures that are effective during the first stages of the fermentation process. Many bacteriocin producing strains have been isolated and these increasing interesting in using these isolates as starter or protective culture in traditional fermented food including Nham. *Lactobacilli* and *Pediococci* that being isolated from sausage frequently produce bacteriocins or bacteriocin-like compounds as shown for *L. sakei*, *L. curvatus*, *L. plantarum*, *L. brevis* and *L. casei* (Leroy et al., 2006).

3.3.3. Starter cultures for a healthier product

Reduction of undesirable compounds which is the point to be considered during strain selection that no undesirable compounds such as toxins, biogenic amines, or D- lactic acid, that could adversely affect health, are formed. The use of

decarboxylase negative starter cultures that are highly competitive and fast acidifiers prevents the growth of biogenic amine producers and leads to end-products nearly free of biogenic amines. In addition, the inoculation of starter strains that possess amine oxidase activity might be a way to further decreasing the amount of biogenic amines produced in Nham (Limsuwan, 2004).

During the last 20 years, much attention has been focused on the use of starter cultures to guarantee safety and standardize product properties, including consistent flavor, color and reduce fermentation time (Baruzzi et al., 2006). The first stage in designing starter culture for fermented meat is to characterize the microorganisms strains isolated from the fermented meat products (Ammor and Mayo, 2007). The most promising bacteria for starter cultures are those which were isolated from the indigenous microflora of traditional products. These microorganisms are well adapted in the meat environment and are capable of dominating the microflora of products (Drosinos et al., 2005).

3.4. Development of starter cultures for Nham fermentation

The use of starter cultures for improving the quality of Nham has received a lot of attention. The development of starter culture involves intense research on the roles of microorganisms during Nham fermentation. The biochemical and physiological roles of these microorganisms in the development of flavor and aroma of the Nham product must be elucidated. This information can be used as the criteria in the selection of starter microorganisms for used in the fermentation. Finally, the starter culture selected would be the one giving a satisfactory performance in the process and also giving an acceptable organoleptic evaluation of the Nham product.

Svetvivadhana (1990) reported the advantage of using mixed culture strains of *Pediococcus* spp. and *Lactobacillus* spp., which gave the highest antagonistic effect on the inoculated salmonellae (*S. anatum*, *S. derby* and *S. newport*) in Nham. Moreover, this study achieved shorter fermentation time and more safety in getting

"*Salmonellae* free products" when using starter cultures in contrast to naturally fermented products. Based on the organoleptic tests carried out by both consumer test panelists and producers, Nham prepared with starter cultures were favorably preferred more than the naturally fermented products.

Wiriyacharee et al., (1990) proposed that *L. plantarum* and *P. cerevisiae* are important for acid production and *M. varians* (reclassified as *K. varians*) produces nitrite reductase which is important in converting nitrate to nitroso-haemoglobin giving pink color to the product. These bacteria were used as starter cultures for producing Nham prduct; and this is the first commercial production of Nham that used starter technology. Krairojananan et al., (1997) found that most of LAB and other aerobic bacteria isolated from Nham produce hydrogen peroxide, a strong oxidizing agent causing discoloration and rancid off-odor. Hence the ability to produce catalase is also a desirable trait for bacterial strains used in Nham fermentation.

Twichatwitayakul, (1996) reported the effect of using mixed starter cultures of *L. plantarum, P. cerevisiae* and *M. varians* on the growth of *Salmonella* (*S.* Typhimurium and *S. anatum*) at 10^3 CFU/g during Nham fermentation. The mixed starter cultures can be used to reduce the growth of *Salmonella* after 5 days of fermentation at a pH of 4.37 and 1.39% of total lactic acid.

Noonpakdee et al., (2003) focused on the isolation of LAB and screening for bacteriocin production. *Lactococcus lactis* strain WNC 20 produced a bacteriocin that not only inhibited closely related LAB, but also *L. monocytogenes, C. perfringens, B. cereus* and *S. aureus.* Thus, *Lc. lactis* strain WNC 20 may be useful in improving the food safety of the fermented food product.

Visessanguan et al., (2006b) reported that Nham inoculated with *L. curvatus* at 10^4 and 10^6 CFU/g exhibited a higher rate of fermentation than natural Nham fermentation based on pH and lactic acid production. In terms of acceptability, Nham inoculated with *L. curvatus* at 10^4 CFU/g received the highest score on flavor, sourness, saltiness, and texture. However, unusual smell was detected in Nham inoculated with *L. curvatus* at 10^6 CFU/g.

Luxananil et al., (2009) and Tosukhowong et al., (2011) studied *L. plantarum* BCC 9546 as a starter culture for Nham to prevent the biogenic amine formation (cadaverine, putrescine, histamine and tyramine) that effects on nervous, blood pressure, gastric and intestinal systems. The results showed the accumulation of tyramine, putrescine, cadaverine, histamine and spermidine were decreased in Nham inoculated with *L. plantarum* BCC 9546 compared to control Nham with no inoculation.

Kingcha et al., (2012) reported that the effect of using starter cultures of *pediocin* PA-1/AcH producing *P. pentosaceus* BCC 3772 on the growth of *L. monocytogenes* with decreased 3.2 logs in population of spiked *L. monocytogenes* within 18-24 h of Nham fermentation.

3.5. Response surface methodology

Determining the optimal conditions for fermentation can be investigated by using the traditional methods and also from the response surface methodology (RSM). However, the use of RSM provides more possibilities to test multiple process variables with fewer experimental trials compared to the study of one variable at a time. Response surface design is the design that uses a full quadratic equation or second-order equation for fitting response surface. Based on RSM, the central composite design (CCD) method is popularly used in the vicinity of the optimum conditions to locate the true optimum values of the multiple variables (Sadik et al., 2007; Tung et al., 2011). Moreover, it can be possible to observe both main effect and interaction of independent variable on responses. The second order model that related the response to independent variables is employed to receive information about the system. However, the disadvantage of RSM is to fit the data to a second order polynomial; and thus some cases, the data could not be curveted. That means there is not fit with this model; and preliminary work becomes more critical for the determination of the independent parameter range.

4. Identification of lactic acid bacteria

Over the past decades, the scientific community has paid special attention to the correct identification of LAB used for food fermentation (Temmerman et al., 2004). Several methods have been developed and applied for identifying LAB. These methods can be used for the screening and selection of LAB, assessing their roles during fermentation and to find potential starters for ensuring safety and stability of fermented products. A broad range of identification techniques are available, all displaying differences in discriminatory power, reproducibility and work load. An overview of phenotypic and genotypic methods that are currently used for identification of LAB as the following provided.

4.1. Phenotypic methods

Traditionally, LAB isolates have been identified on the basis of phenotypic methods. These methods include morphology physiology and biochemistry, particularly carbohydrate fermentation patterns (Gonzalez et al., 2000). Phenotypic tests are still being used on a routine basis for the identification of food-associated LAB. The popularity of these methods is mainly due to the fact that no specialized equipment is required to carry out most tests and because of the availability of an identification database (Temmerman et al., 2004). Additionally, phenotypic methods are less expensive and require less training for lab personnel compared to genetic methods.

4.1.1. Morphological and cultural characteristics

In general pediococci, streptococci, enterococccci and lactococci can be differentiated morphologically, in contrast to lactobacilli and carnobacteria. However, all of them have similar colonial appearance (Kunawasen, 2000). Drosinos et al., (2005) identified 288 LAB isolates from a traditional Greek fermented sausage using

cell morphology and physiology characteristics followed by further identification using the commercial API 50 CHL system (BioMérieux, France). These studies demonstrated that cell morphology gave several doubtful and low discrimination profiles.

4.1.2. Physiological characteristics

The ability to grow at various conditions (presence or absence of oxygen, pH, temperature, etc.) can discriminate among genera of LAB. For example, discrimination between *Lactobacillus* and *Carnobacterium* can readily be achieved because the latter can not grow at pH 4.5 or on acetate agar but can grow at pH 9.0 (Kunawasen, 2000). Gonzalez et al., (2000) identified 249 LAB isolates from freshwater, fish and their environments using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level, demonstrating the low taxonomic resolution of this labor-intensive approach.

4.1.3. Carbohydrate fermentation patterns

The determination of the carbohydrate fermentation pattern using conventional test tube techniques is subject to immense variation, depending on the procedures used (Kunawasen, 2000). API (BioMérieux) and BIOLOG system are the most popular commercially available miniaturized identification systems (Temmerman et al., 2004; Ashraf et al., 2009). The determination of carbohydrate fermentation is not very convenient and may be misleading (Shaw and Harding, 1984). Furthermore, it is unsatisfactory to consider only carbohydrate fermentation patterns because variable fermentations often occur (Champomier at el., 1987). Andrigetto et al., (1998) found that some strains were falsely assigned to species or subspecies on the basis of sugar fermentation profiles obtained by API 50 CHL. Although the application of phenotypic techniques has proven to be useful for certain LAB, these identification methods still have their limitations. Because of relatively poor reproducibility and low taxonomic resolution that often only allows differentiation at the genus level (Temmerman et al., 2004). The shortcomings of phenotypically based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence (Olive and Bean, 1999).

4.2. Genotypic methods

Molecular techniques have been successfully applied for LAB identification which permitted accurate and fast identification (Cocolin et al., 2004; Ammor et al., 2005; Iacumin et al., 2006; Kostinek et al., 2007). Genotypic methods exhibit various levels of discriminatory power, from species level to strains level. In recent years, several molecular methods have been used for differentiating LAB such as restriction fragment length polymorphism (RFLP) (Temmerman et al., 2004), pulsed-field gel electrophoresis (PFGE) (Tran et al., 2011), ribotyping and DNA-DNA hybridization (Sato et al., 2001). Furthermore, many genotypic methods are based on the principle of polymerase chain reaction (PCR) including amplified fragment length polymorphism (AFLP) (Temmerman et al., 2004), random amplified polymorphic DNA (RAPD) (Ammor et al., 2005), 16S rDNA sequence, 16S-23S rDNA Internal Transcribed Spacer (ITS)-PCR (Song et al., 2000), rep-PCR (Gevers et al., 2001) and PCR-denaturing gradient gel electrophoresis (DGGE) (Albano et al., 2008). Each technique is described in more detail as follows.

4.2.1. Restriction fragment length polymorphism (RFLP)

This technique involved the digestion of genomic DNA with restriction enzymes, followed by electrophoresis, southern transfer, radioactive or non-radioactive probing and exposure of the filters to film. This restriction endonuclease analysis based on sequence data of those fragments was used for differentiation among species (Kunawasen, 2000). In addition, the discriminatory power of these methods is very high (i.e. strain level) making them very useful for typing LAB starter cultures, of which the strain-specific properties are crucial to the production process (Temmerman et al., 2004).

4.2.2. Ribotyping

Ribotyping combines an enzymatic restriction digest with the detection of the resulting fragments by means of rDNA probes. Either fluorescent or radioactively labeled probes can be used to hybridize with specific DNA sequences (Temmerman et al., 2004). Ribotyping has been applied with success for the identification of LAB from vacuum-packaged 'gravad' rainbow trout (Lyhs et al., 2002) and morcilla de Burgos (Santos et al., 2005). However, ribotyping provides high discriminatory power at the species and subspecies level rather than on the strain level (Ammor et al., 2005).

4.2.3. Pulsed Field Gel Electrophoresis (PFGE)

PFGE employs an alternating field of electrophoresis to allow separation of the large DNA fragment obtained from restriction digest with rare cutting enzymes (Temmerman et al., 2004). PFGE is the most discriminatory power and reproducible method for differentiating lactobacilli at the strain level (Plengvidhya et al., 2004; Drosinos et al., 2005). PFGE is often considered the "gold standard" of molecular typing methods (Olive and Bean, 1999). However, it is a laborious and expensive method, with only a limited number of samples can be analyzed at the same time. Moreover, performing PFGE is very time-consuming and not suited for a routine use in many laboratories.

4.2.4. DNA-DNA hybridization

DNA-DNA hybridization has higher resolution than 16S rDNA sequencing (Temmerman et al., 2004). Various approaches exist such as the nitrocellulose filter methods, free-solution methods, and more recently the use of microarray technology (Cho and Tiedje, 2001). DNA-DNA hybridization was used to select probiotic species out of 297 *Lactobacillus* isolates (Du Toit et al., 1998). Although the DNA-DNA hybridization technique has improved knowledge on taxonomic relationships between *Lactobacillus* species, but this technique is still time-consuming and labor-intensive (Kwon et al., 2004)

4.2.5. Amplified fragment length polymorphism (AFLP)

AFLP is a genome fingerprinting technique that combines PCR amplification with double enzyme restriction digestion. Total genomic DNA is digested using two restriction enzymes and double-stranded adapters are usually ligated to the DNA fragments serving as primer binding sites for PCR amplification. The AFLP procedure is more labor-intensive than Rep-PCR, but results are obtained more rapidly than with PFGE (Olive and Bean, 1999). AFLP has been found to be a very useful fingerprinting technique for bacteria, allowing both species resolution and strain differentiation (Temmerman et al., 2004).

4.2.6. Random amplified polymorphic DNA (RAPD)

RAPD is based on the use of arbitrary primers, 9 to 10 base in length which hybridized with sufficient affinity on chromosomal DNA sequences at low annealing temperatures that they are able to bind under low stringency to a number of partially complementary sequences of unknown location in the genome of an organism. The number and location of these random primer sites vary for different strains of a bacterial species. Therefore, following separation of the amplification products by agarose gel electrophoresis a pattern of bands, which in theory is characteristic of the particular bacterial strain results (Olive and Bean, 1999). RAPD has been widely reported as a rapid, sensitive, and inexpensive method for genetic typing of different strains of LAB and bifidobacteria (Ammor et al., 2005). However, primer with a high discriminatory power and a broad applicability within a large group of LAB species level is not available. Moreover, the resulting band patterns are often exhibit a poor reproducibility because RAPD primers are not directed against a particular genetic locus, thus many of priming events are the result of imperfect hybridization between primer and the target site (Olive and Bean, 1999; Gevers et al., 2001). RAPD was more discriminating than RFLP analysis of 16S rRNA gene or the 16S-23S rRNA spacer region but less discriminating than Rep-PCR (Olive and Bean, 1999).

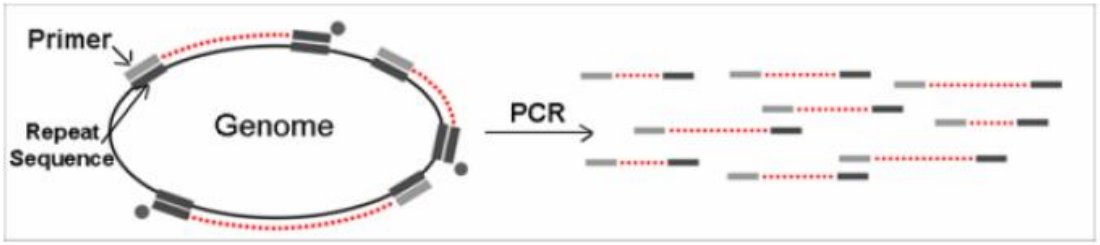
4.2.7. Repetitive DNA element -based PCR (Rep-PCR)

Rep-PCR genomic fingerprinting developed by Versalovic et al., (1991) is currently used to estimate relative degrees of similarity between different isolates and to generate species-specific fingerprint. Depending on the type of primer such as REP, ERIC, BOX or (GTG) 5 used and on the conditions of the reaction (Fani et al., 1993; Versalovic et al., 1994; Olive and Bean, 1999); this technique is easy to perform and can be applied to a large number of isolates. In addition, rep-PCR has a high discriminatory power, low cost to perform making this technique suitable for a high-through put of strains and considerable to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Gevers et al., 2001). Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or RAPD. Rep-PCR has considerably better discriminatory power than restriction analysis of the 16S rRNA gene or the 16S-23S spacer region (Temmerman et al., 2004). Furthermore, studies which compared Rep-PCR to other typing methods such as multilocus enzyme electrophoresis, biochemical characterizations, or ribotyping have shown Rep PCR to be superior to these methods (Ammor et al., 2005). Finally studies have shown Rep PCR to have a good correlation with PFGE but, in general, with slightly less discriminatory power (Olive and Bean, 1999; Tran et al., 2011).

Repeated sequences are present in the genomes of all organisms. The first described repeated sequences in prokaryotes and may be the most extensively studied is the repetitive extragenic palindrome (REP) or palindromic unit (PU) sequence initially identified in *Salmonella typhimurium* and *E. coli*. The repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock, 1992). Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic et al., 1994). These sequences appear to be located in distinct, intergenic positions around the genome. Oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of

BOX, in PCR (Versalovic et al., 1994). The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprint. Rep-PCR genomic fingerprinting was found to be extremely reliable, reproducible, rapid and highly discriminatory (Versalovic et al., 1994). The process known as repetitive sequence-based PCR (rep-PCR), allows for the amplification of many different sized fragments (amplicons) representing the DNA within the non-coding, repetitive sequences in the genome (Figure 2.7).

Step 1 rep-PCR primers bind to many specific repetitive sequences interspersed throughout the genome. Multiple fragments of various lengths are amplified.



Step 2 Fragments can be separated by size and charge. A unique rep-PCR fingerprint profile is created containing multiple bands of varying sizes and intensities.

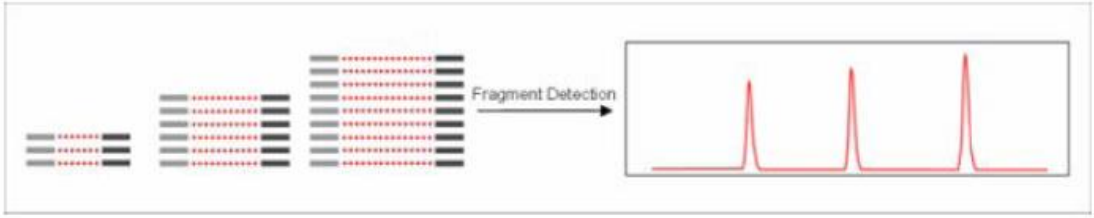


Figure 2.7. Repetitive sequence-based PCR (Rep-PCR) steps.

Source: Versalovic et al., (1994)

4.2.8. 16S rDNA sequencing

DNA sequencing generally begins with PCR amplification of a sample DNA directed at genetic regions of interest, followed by sequencing reactions with the PCR

products. The obtain sequence is to be compared with DNA sequences stored in online databases of previously sequence DNA, of which the most popular ones are the EMBL (http://www.ebi.ac.uk/embl) and Genbank (http://www.ncbi.nlm.nih.gov/Genebank/) databases. The 16S rRNA-targeted hybridization probes and PCR primers have been successfully used to identify and detect of some *Lactobacillus* species. However, among closely related species, 16S rRNA gene probes or primers have limited success when using in identification of little variation of the 16S rRNA sequence (Song et al., 2000). In general, DNA sequencing is expensive and requires a high degree of technical competency to perform (Olive and Bean, 1999). Although a very powerful tool, the reliability and the taxonomic coverage the technique are rely on the availability of the database. Furthermore, the degree of strain and inter-operon sequence variation may in some cases yield confusing identification results (Temmerman et al., 2004).

4.2.9. 16S-23S rDNA Internal Transcribed Spacer (ITS)-PCR

The sequence of the 16S-23S rRNA intergenic spacer region exhibits greater variations than that of 16S rRNA structural gene. The amplification of the 16S-23S rRNA intergenic spacer region prove to be a useful alternative to 16S rDNA sequencing for designing specific primers or probes to identify closely related species such as *L. curvatus* and *L. casei* or *L. acidophilus* and *L. helveticus*; (Nour, 1998; Song et al., 2000). Recently, length, sequence variation and RFLP analysis of the 16S-23S rDNA spacer regions have been used to provide adequate discriminatory of species in which 16S rDNA sequence are similar (Laganowska and Kaznowski, 2004).

The spacer regions of ribosomal operon are transcribed, and for this reason they are called intergenic transcribed spacer or internal transcribed spacer (ITS) regions (Përez-luz et al., 2002). De Vries et al., (2006) reported the ITS between the 16S and 23 S genes contain between zero and two tRNA genes, usually encoding a tRNA-Ile, tRNA-Ala or tRNA-Glu (Figure 2.8). The amplification of the 16S-23S rDNA spacer region and the size of polymorphism of the resulting PCR products have

been successfully used for the species-level differentiation and identification of a broad range of bacteria (Gürtler and Stanisich, 1996; Mendoza et al., 1998; Riffard et al., 1998; Christensen et al., 1999) including of *Lactobacillus* species (Tannock et al., 1999; Song et al., 2000).

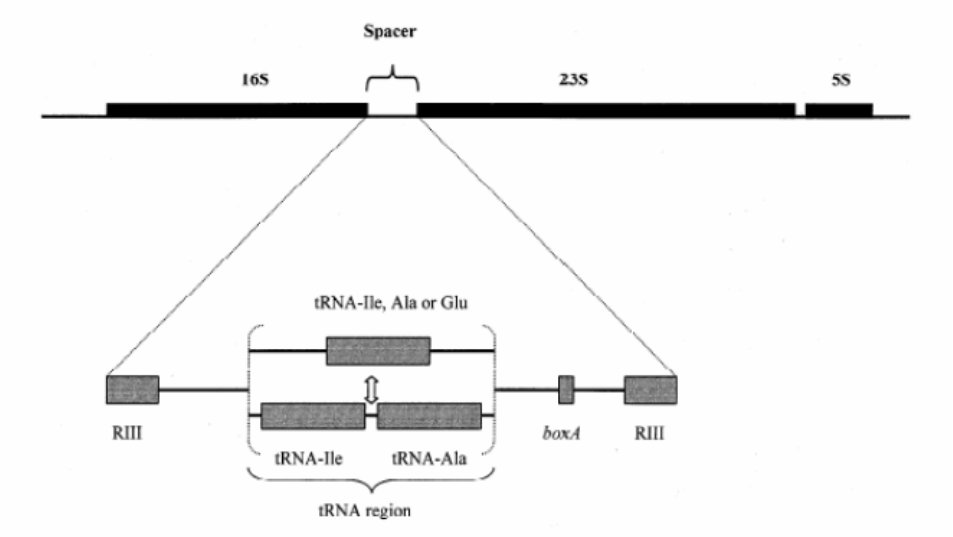


Figure 2.8. Schematic representation of a 16S–23S spacer and organization of its functional regions (shadowed boxes). As indicated by brackets, the presence of tRNA genes is not universal and their number and type may vary among species

Source: García-Martínez et al., (1999)

4.2.10. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the nonculture-dependent fingerprinting techniques (Cocolin et al., 2011). The technique is based on the electrophoretic separation of PCR-generated double stranded DNA in a polyacrylamide gel containing a gradient of chemical denaturants (urea and formamide). As the DNA molecule encounters an appropriate denaturant concentration, a sequence-dependent, partial denaturation of the double strand occurs. This change in the conformation of the DNA structure causes a reduced migration rate of the molecule. In the temperature gradient gel

electrophoresis (TGGE), the temperature is the main denaturing agent. When the method is used for microbial profiling, DNA and/or RNA are subjected to PCR and/or Reverse Transcription (RT)-PCR with universal primers, able to prime amplification for all the microbes present in the sample. After this step, the complex mixture of the DNA molecules obtained can be differentiated and characterized if separated in denaturing gradient gels. Every single band that is visible in D/TGGE gels represents a component of the microbiota. The more bands are visible, the more complex is the ecosystem. Bands can be excised from the gels and after re-amplification can be sequenced in order to obtain the corresponding microbial species. By using these methods, it is possible not only to profile the microbial populations, but also to follow their dynamics during time.

The application of PCR–DGGE in the field of fermented sausages offers a better understanding of the biodiversity and dynamics of the populations involved in the transformation. However, it should be mentioned that pitfalls, associated with sampling, DNA extraction, DNA purity, PCR conditions, formation of heteroduplex and chimeric molecules, may still exist, thereby the results obtained need to be verified and validated (Ercolini, 2004). One important aspect that has to be taken into consideration when applying DGGE in food fermentation is the sensitivity limit. It has been demonstrated that populations that are below 10^3–10^4 CFU/g will not be detected (Cocolin et al., 2001). This is especially valid when in the same ecosystem has two populations, one at high and the other at low counts, exist, as it usually happens in sausage fermentations. Moreover, due to the extensive application of sequencing, databases have seen tremendous growth of the sequences deposited. Often, these entries are classified as ‘unculturable microorganism’, since they have been detected only by culture-independent methods and no significant similarity to available sequences was obtained. This aspect introduces potential difficulties in the understanding of the ecology of fermented foods and at the same time underlines the need to improve the traditional cultivation methods.

CHAPTER 3

MATERIALS AND METHODS

1. Isolation and selection of LAB to be used as starter cultures

1.1. Isolation of LAB from fermented foods

Lactobacilli MRS broth (de Man Rogosa and Sharpe: MRS) was used for isolation, selection and maintenance of LAB strains. Supplementation with 0.5% monosodium glutamate (MSG) into MRS broth to help induce GABA production by isolated LAB was a modified medium used in this study. One hundred and sixty three samples of various Thai fermented foods were purchased from local markets in southern Thailand. In the case of fermented food samples in a form of solid, 50 g of each sample was added into 450 mL of normal saline (0.85% NaCl) and mixed well by using a stomacher for 30 sec. The suspension was streaked onto MRS agar and incubated at 30^{o}C for 18 h. Fermented food samples in a liquid form were directly streaked as one sample per plate. To indicate acid producing by bacteria, 0.04% bromocresol purple was added into the MRS agar and colonies with a yellow zone around them were isolated. After purification, each isolate was initially examined for catalase production and if no producing of catalase was detected, it meant that the isolate was most likely a lactic acid bacterium. The isolated strains were stored at -20^{o}C in 20% glycerol. Pure culture was activated in stab MRS agar by incubating at 30^{o}C for 18 h prior to use for further studies.

1.2. Screening of GABA-producing LAB

Two steps were conducted to select GABA-producing LAB. First primary screening based on the ability to grow well in MRS broth by growing 1 loopful of each culture in 5 mL MRS broth in a test tube and all culture tubes were incubated under static condition at 30^{o}C for 18 h. Bacterial growth was measured by a spectrophotometer at OD_{660} nm and any isolates that gave OD_{660} > 1.0 were selected for further studies. Secondary screening was carried out to select GABA-producing LAB. To prepare the inoculum, one loopful of stab culture was inoculated into 10 mL MRS medium in a test tube and incubated as previously described. Each culture broth was then inoculated at 1% (v/v) into a 10 mL fermentation medium containing 0.5% MSG in a test tube and cultivated at the same condition as previously stated for 24 h. Culture broth was centrifuged at 6,359 x *g* for 15 min at 4^{o}C; and GABA presence in culture supernatant was analyzed using thin-layer chromatography (TLC) according to the method as described by Choi et al., (2006). Consequently, those producing GABA were checked for the amount of GABA by HPLC following the methods as described by Ratanaburee et al., (2011a).

1.3. Determination of antibacterial activity

The agar well diffusion assay was used to investigate the antibacterial activity of selected LAB strains. The following food-borne bacteria; *B. cereus* ATCC 11778, *C. perfringens* DMST 16637, *L. monocytogenes* DMST 4553, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Salmonella Typhi* PSSCMI 0034 and *S. sonneii* PSSCMI 0032 were used as the target organisms. All, except for *E. coli* ATCC 25922, were potential food borne pathogenic bacteria. They were all cultured in Tryptic soy agar (TSA) for 24 h at 37°C and one loopful of each culture was inoculated into Tryptic soy broth (TSB) for 4 h. Each culture broth was adjusted to 0.5 Mcfarland units (roughly 1.5 x 10^8 cells/mL) and then diluted to 10^6 cells/mL by sterile normal saline

solution for swabbing onto each TSA plate. The selected LAB strains were separately grown in MRS broth and incubated at 37°C for 48 h. Culture broths were measured pH and determined total acidity as lactic acid, according to the methods of AOAC (2002). A cell-free supernatant was obtained by centrifugation at 6,359 *x g* for 15 min, followed by filtration through a 0.45 μm cellulose acetate filter. A 140 μL sample of each culture filtrate was then added to each well of the TSA plates that were swabbed with target organisms as previously described and all plates were incubated at 37°C for 48 h. After incubation, the inhibition zone diameters were measured for interpreting their antibacterial activity.

1.4. Identification of selected LAB

The following LAB strains; HN8, NH102, NH116 and NH2 were selected for further studies, due to their ability to produce high amount of GABA and showed strong antibacterial activity. They were identified using conventional methods and also using 16S rDNA sequence analysis. Biochemical and physiological properties were tested according to Bergey's Manual of Systematic Bacteriology volume 2 (Garvie, 1986; Kandler and Weiss, 1986). For the molecular technique, the bacteria were grown in MRS broth at 37°C for 18 h. Bacterial cells were harvested for genomic DNA extraction according to Ausubel et al., (2002). The method included enzymatic and SDS treatments, that were conducted first with 50 mg/mL lysozyme and then 20 mg/mL proteinase K then treated with 10% SDS and 100 mg/mL RNase, all suspended in TE buffer. After incubation at 37°C for 60 min, 10% CTAB was added and incubated at 65°C for 10 min and then an equal amount of chloroform was added and gently mixed then centrifuged at 17,211 *x* g for 5 min. The supernatant was taken out into a new tube and gently mixed with an equal volume of phenol/ chloroform then centrifuged. DNA was precipitated from the supernatant with 1 volume of isopropanol and the pellet was washed twice with 70% ethanol.

The 16S-rRNA genes were amplified by PCR using a pair of primers that targeted conserved regions of LAB 16S-rDNA corresponding to position 27F (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1389R (5'-ACGGGCGGTGTG TACAAG-3'). PCR amplification was conducted in a final volume of 50 μL comprising 19.75 μL deionized water, 2.5 μL (each primer), 5 μL 10x PCR buffer (500 mM KCl and 100 mM Tris–Cl, pH 8.0), 5 μL 25 mM $MgCl_2$, 0.25 μL Taq DNA polymerase, 5 μL dNTPs mixture (2 mM each dNTP), 10 μL DNA template (~50 ng/μL). PCR amplification was performed in a GeneAmp PCR, model 9700 (PerkinElmer, Waltham, MA, USA). The amplification conditions were as follows; initial denaturation at 94°C for 3 min, followed by thirty-five cycles of denaturation at 94°C for 30 s, the annealing temperature of the primers was 55°C for 30 s and the extension at 72°C for 2 min. The final extension cycle was 72°C for 5 min and samples then cooled to 4°C. The PCR products were analyzed using gel electrophoresis on 1.5% (w/v) agarose in 1x TBE at 100 V for 30 min. The gels were stained in ethidium bromide and observed on a Geldoc/UV transilluminator. The purified PCR products were sequenced with primer 520F 5'-CAGC(A/C)GCCGCGG TAAT(A/T)C-3' using an automate DNA sequencer (3100-Avant Genetic Analyzer, ABI, Carlsbad, CA, USA). The partial 16S rDNA sequence was compared with the GenBank database in the NCBI website (htpp://www.ncbi.nlm.nih.gov/Blast) using blastn. CLUSTAL X 2.0 (Larkin et al., 2007) was used for multiple alignments, 4 sample strains from this experiment had been computed along with other strains of closely related LAB available from the GenBank database and *L. monocytogenes* ATCC 19115^T (EU295337) was also added as an out-group. Phylogenetic tree was performed using MEGA4 (Tamura et al., 2007). The evolutionary distance matrix was calculated according to Jukes and Cantor method and unweighted pair-group method with arithmetic averages augorithm was chosen for clustering.

1.5. Testing of probiotic properties *in vitro*

1.5.1 Biological barriers

Survival of 4 selected LAB strains under simulated stomach conditions was tested according to their acid tolerance as described by Vinderola and Reinheimer (2003). Each culture grown in MRS broth at 37°C for 24 h was harvested by centrifugation at 6,359 x *g* for 15 min at 4°C. Cell pellets were washed twice with phosphate buffer saline (PBS) buffer, pH 7.4 and then resuspended in PBS solution by adjusting the pH to 2.0 and adding 3 mg/mL pepsin. The cell suspension was incubated at 37°C for 2 h and the isolates tolerance was assessed in terms of a viable colony count on MRS agar.

The challenged LAB strains were then centrifuged and washed twice with PBS solution. Each cell pellet was resuspended in PBS solution, pH 8.0 containing 1 mg/mL pancreatin and 3 mg/mL ox bile salts for 2, 4 and 6 h at 37°C (Maragkoudakis et al., 2006). Tolerant LAB strains were again assessed in terms of viable colony counts on MRS agar. This represents survival in the stomach and intestine; hence, allows them to reach the colon.

Growth of selected LAB with aerobic and anaerobic conditions was carried out according to Talwalkar et al., (2001) by adding 1% of a 24 h LAB culture into 10 mL MRS broth (aerobic conditions) and 10 mL MRS broth containing 0.5 mg/mL L-cysteine (anaerobic conditions). The culture tubes were incubated under static condition at 37°C for 24 h. The oxygen concentration was measured in the form of an oxidation–reduction potential (ORP: Eh). The ORP probe was used to monitor the Eh values in the inoculated medium; and the data were recorded after obtaining a constant value. The probe was frequently checked in a quinhydrone buffer solution following the method specified by the manufacturer. Before each run, the electrodes were fully washed with sterile distilled water. For aerobic conditions, cells were counted on MRS agar and incubated at 37°C for 24 h. For checking growth in anaerobic conditions, cells were counted on MRS agar containing 0.5 mg/mL L-cysteine and overlaid with 15 g/L agar, followed by incubation at 37°C for 24 h in an anaerobic jar.

1.5.2. Cell surface hydrophobicity

In this study, bacterial adhesion was determined *in vitro* using the hexadecane droplets, as this method was reported to be a qualitatively valid method to estimate the ability of a strain to adhere to epithelial cells because it was a measure of their surface hydrophobicity (Ram and Chander, 2003). Cultures grown in MRS broth at 37°C for 24 h were harvested by centrifugation at 6,359 x *g* for 15 min. After being washed twice with PBS, pH 7.4, cells were resuspended in the same buffer by adjustment to an optical density (OD_{560} nm) of 0.8–1.0 (control) spectrophotometrically. Then, 0.6 mL of n-hexadecane was added into 3 mL of a bacterial suspension and mixed using a vortex mixer for 2 min. The tubes were allowed to stand at 37°C for 1 h to separate the two phases. The aqueous phase was carefully removed and the OD_{560} nm of the aqueous phase was measured. The percentage of cell surface hydrophobicity of the strain adhering to hexadecane was calculated by comparing with the control.

1.5.3. Blood haemolysis

Blood haemolysis of selected LAB strains was investigated by growing the organism in MRS broth at 37°C for 18 h and then transferring onto blood agar plates. After 48 h; any haemolytic reaction was observed and recorded as follows: a partial hydrolysis of red blood cells showing a greening zone (α-haemolysis), a clear zone around bacterial growth (β-haemolysis), and no reaction (γ-haemolysis).

1.5.4. Bile salt hydrolase activity (BSH)

A qualitative test of each isolate for BSH activity was performed on MRS agar containing 0.5% (w/v) of human bile salts as follows: glycocholic acid (GC),

taurocholic acid (TC), glycodeoxycholic acid (GDC) and taurodeoxycholic acids (TDC) (Noriega et al., 2006). Overnight cultures of each LAB strain (10 μL) were spotted onto the agar plates and incubated at 37°C for 24 h. The presence of precipitated bile acid around the colonies (opaque or halo) was considered to be a positive result.

1.5.5. Removal of cholesterol

To test the cholesterol-lowering property of LAB, the method of Sirilun et al., (2010) was adopted with some modification as follows. A 10 mL of MRS broth was supplemented with 0.3% ox gall, as the bile salt, and then a filter-sterilized cholesterol solution (10 mg/mL in ethanol) was added to make a final concentration of 70 μg/mL. An uninoculated sterile MRS broth was used as a control set. Cells of individual strains grown in the above culture broth under anaerobic conditions using a GasPak anaerobic jar for 24 h at 37°C were removed by centrifugating at 11,952 *xg*, 4°C for 7 min. The cholesterol remaining in each culture supernatant was determined by adding 0.5 mL into a clean test tube followed by 3 mL of 95% ethanol and then 2 mL of 50% potassium hydroxide. After being well mixed, all tubes were heated in a 60°C water bath for 10 min and after cooling, 5 mL of hexane and 3 mL of distilled water were added into each tube. After incubating at room temperature for 15 min to allow phase separation, 2.5 mL of the hexane layer was transferred into a clean test tube for evaporation at 60°C and then adding 4 mL of O-phthalaldehyde reagent into the tube and incubating for 10 min. Then, 2 mL of concentrated sulphuric acid was slowly pipetted down the side of the tube with immediate thorough mixing and after standing at room temperature for 10 min, the OD_{620} nm was read. The amount of cholesterol was determined from the standard curve and ability of bacterial cells to remove cholesterol was calculated by comparison with a control set.

1.5.6. Antibiotic susceptibility

An agar disc diffusion method was used to test the antibiotic susceptibility of selected LAB strains with 15 commonly used antibiotics as follows: ampicillin (10 µg), bacitracin (10 µg), cefoperazone (75 µg), ceftazidime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), norfloxacin (10 µg), pencillin G (10 µg), polymyxin B (300 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). An active culture of each LAB strain was prepared to obtain a cell suspension of 10^6 cells/mL as previously described. The bacterial suspension was swabbed onto the agar plates in three directions over the entire surface; later different antibiotic discs were placed on the agar and incubated at 37°C for 24 h. The diameters of the inhibition zones were measured after incubation and compared with those in the interpretative standard chart (National Committee for Clinical Laboratory Standards (NCCLS), 2004). The results were reported as resistance (R), intermediate (I) and susceptible (S).

2. Use of starter cultures for producing Nham

2.1. Optimization of the key factors to produce GABA Nham

2.1.1. Experimental design

The table salt NaCl is used for Nham production and its concentration varies in the range of 2.00 to 2.50% (w/w). Addition of MSG to Nham for enhancing GABA production was varied from 0 to 0.50% while the cell density for inoculation was varied over a range of 6-8 log CFU/g (wet weight). The optimal combination of starter cultures, NaCl and MSG for producing GABA in Nham were designed using different amounts of starter cultures: X_1 (log CFU/g), % NaCl: X_2 (w/w) and % MSG:

X_3 (w/w) by the CCD with three levels. The behavior of the system was explained by the following quadratic equation as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_j^2 \quad \text{(Eq.1)}$$

where Y is the predicted response, β_0 the offset term, β_i the linear offset, β_{ij} the interaction effect, β_{ii} the squared offset, and Xi-Xj are the independent variables. Various assemblies used in the design are shown in Table 3.1. The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 6.0.2 Trial, Stat-Ease Corporation, USA). The optimal fermentation conditions for producing GABA was achieved by solving the regression equations including analyzing the response surface contour plots using the same software. The significances of all terms in the polynomial were judged statistically by computing the *F*-value at a probability level (*P*) of 0.01 or 0.05. The model was then resubmitted for statistical analysis to neglect all terms that are statistically insignificant ($P>0.05$). Regression coefficients were used to generate a contour map for the regression model and the quality of the fit of the model equations were expressed by the coefficient of determination, R^2.

Table 3.1 Experimental design using the Central Composite Design on the GABA content in Nham.

Run number	Starter (X_1)[a] (Log CFU/g)	NaCl (X_2) % (w/w)	MSG (X_3) % (w/w)
1	6.00	2.00	0.00
2	8.00	2.00	0.00
3	6.00	2.50	0.00
4	8.00	2.50	0.00
5	6.00	2.00	0.50
6	8.00	2.00	0.50
7	6.00	2.50	0.50
8	8.00	2.50	0.50
9	6.00	2.25	0.25
10	8.00	2.25	0.25
11	7.00	2.00	0.25
12	7.00	2.50	0.25
13	7.00	2.25	0.00
14	7.00	2.25	0.50
15	7.00	2.25	0.25
16	7.00	2.25	0.25
17	7.00	2.25	0.25
18	7.00	2.25	0.25
19	7.00	2.25	0.25
20	7.00	2.25	0.25

[a] X_1 log CFU/g for each strain

2.1.2. Preparation of starter cultures

P. pentosaceus HN8 and *L. namurensis* NH2 are the GABA producing LAB previously isolated from fermented meats that were used as starter cultures for

producing Nham. The former organism was found to be the best GABA producer while the latter organism produced less acid and thus could reduce post acidification of Nham, and therefore prolong the shelf life of the finished product (Ratanaburee et al., 2013a, b). One loopful of each starter culture kept in MRS broth with 20% glycerol at -80°C was cross-streaked onto MRS agar and incubated at 30°C for 48 h. A single colony of each LAB on the MRS agar was transferred into 10 mL of MRS broth and incubated at 30°C for 18-24 h. The bacterial cells were harvested by centrifugation at 4°C at 6,359 x*g* for 15 min. The harvested cells were washed twice with 10 mL of sterile 0.85% NaCl solution and finally cells were adjusted with sterile 0.85% NaCl to a density of 10^9 CFU/mL.

2.1.3. Nham Production

Ground pork was purchased from a local retailer and all visible fat and connective tissues were trimmed followed by processing into Nham. Briefly, Nham products were prepared according to the traditional technique by mixing ground pork (60%, w/w), salt, starter cultures (X_1 log CFU/g for each strain) and MSG as indicated in Table 3.1 with the following ingredients: cooked sticky rice, cooked pork rind, garlic, table sugar, potassium nitrate, pepper, and bird chilli. The amount of each ingredient is not shown as it is a commercial secret. The Nham products were extruded through a stuffing horn into plastic casings with a diameter of 3.0 cm (approximately 50 g each) and tightly sealed. All products in triplicate were fermented by incubation for 96 h at 30°C. Nham products were randomly sampled for determining their GABA content at 0 h (soon after the mixture was stuffed into the casing) and at 96 h. Nham samples were extracted to release GABA using 4% acetic acid (Nham and 4% acetic acid with a ratio of 1: 4 w/v). The amount of GABA in the Nham was determined by HPLC according to the method of Ratanaburee et al., (2011a). All actual amounts of GABA were used to analyze for the predicted values of GABA using Equation 1 and the optimal concentrations of NaCl, MSG and inoculum for producing GABA was achieved as previously described in 2.1.

Verification of the test after obtaining the optimum conditions was also carried out to confirm the results.

2.2. Characterization of GABA Nham and fermentation

The recipe described in 2.1.3 that produced Nham with the highest amount of GABA is called GABA Nham. Properties of the GABA Nham and the fermentation process including sensory tests were investigated by comparison to controls to understand better the effects of the starter cultures and MSG. Hence, four processes for producing Nham were compared. There were: (1) without inoculation of starter cultures and no addition of MSG (spontaneous Nham: T_{NN}: Control A); (2) with only the addition of 0.5% MSG (Control B: T_{NM}); (3) with only the inoculation starter cultures (roughly 10^6 CFU/g of each by 1: 1, T_{SN}) and (4) with both additions of starter cultures and 0.5% MSG (GABA Nham, T_{SM}). Nham products were sampled at the beginning of the fermentation and every 24 h for 96 h to characterize their properties, microbial populations and sensory tests were carried out as described below.

2.2.1. Microbiological changes

Samples were collected aseptically and used as the composite sample. The composite sample (25 g) was placed in a stomacher bag containing 225 mL of 0.85% NaCl. After mixing for 1 min in a stomacher blender (Stomacher M400, Seward Ltd., Worthington, England), further serial dilutions were prepared from this homogenate using 0.85% NaCl as the diluent. Appropriate dilutions were used for microbial enumerations. A total bacterial count (TBC) and LAB were determined by the pour plate method on Plate Count Agar (PCA) and de Man Rogosa Sharpe (MRS) agar plates, respectively. All plates were incubated at 30°C for 24-48 h. Staphylococci

were grown on Baird Parker agar plates and incubated at 30°C for 24-48 h. Black colonies, due to the precipitation of ferrous sulfide on this medium, were counted. For the determination of the yeasts, 0.1 mL of an appropriately diluted homogenate was spread on potato dextrose agar (PDA) plates and incubated at 30°C for 48-72 h.

2.2.2. Physicochemical changes

Total acidity was measured by the titration method (AOAC, 2000). Each sample (5 g) was homogenized in 50 mL of distilled water at 6,359 x*g* for 1 min using an Ultra Turrax homogeniser (IKA Labortechnik, Selangor, Malaysia). The supernatant was filtered through a filter paper (Whatman No. 4) (Whatman International Ltd., Maidstone, England). The filtrate was titrated with standardized 0.1 N NaOH. The total acidity in the sample was expressed as the percentage lactic acid based on its dry weight. To measure the pH, a sample (2 g) was homogenized with 10 volumes of deionized water for 1 min and the homogenate was kept at room temperature for 5 min before measuring the pH (Visessanguan et al., 2004). The pH was determined using a pH-meter (Mettler Teledo 320, Switzerland).

The amount of GABA in Nham was determined at the beginning and at the end of the fermentation by following the method as previously described. The proximate composition was determined according to the AOAC (2000) methods. The moisture content was determined by drying the samples overnight at 100^{o}C until a constant weight was achieved. Crude protein content was determined using the Kjeldahl method while crude lipid content was determined using the acid hydrolysis method. The fiber content was determined using the Soxhlet method; and the ash content was determined by ashing samples overnight at 550^{o}C. The carbohydrate content was calculated by difference.

A texture profile analysis (TPA) of all Nham samples based on their firmness (hardness) and toughness was carried out using a model TA-XT2 texture analyzer (Stable Micro System, Surrey, UK). Samples were equilibrated at room temperature

(25-30ºC) for 2 h before analysis. Ten cylindrical samples (2.5 cm in length) were prepared and tested. The breaking force (strength) and deformation (cohesiveness/ elasticity) were measured by the texture analyzer equipped with a spherical plunger (5 mm diameter) with a cross-head speed of 60 mm/min and 60% compression. The Stable Micro system and the Texture Expert version 1.0 software were used to collect data for calculation of the total work in Newton. TPA parameters; hardness was calculated from the force-time curves generated for each sample by following the method as described by Bourne (1976) while toughness was calculated from the force–deformation curves according to the method of Rao and Gault (1990).

Weight loss and released water were determined according to the methods as described by Nakao et al., (1991). To determine the weight loss, Nham with casing (100 g) was weighed accurately before fermentation using a balance. During the fermentation process, Nham was reweighed. The difference in weight of Nham before and after fermentation was referred to as the weight loss. The water released from samples was measured immediately upon collecting the sample. The sample with casing was weighed (A). After removing the sample from the casings, water released on the surface was absorbed using filter paper and the sample was reweighed (B). Finally, the empty casing was weighed (C). The percentage of released water was calculated according to the equation as follows:

$$\text{Released water (g/100 g)} = \{100 \times (A-B) - C\}/(A-C)$$

Water activity was measured by a water activity meter (AquaLab, Model 3TE, Decagon Devices, USA) using a 5 g undiluted sample following the instructions for the equipment.

The color of the samples was measured by a Minolta color meter (Color Meter CR-300, Minolta Camera Ltd., Osaka, Japan) and was reported by the CIE system. L*, a* and b* are parameters that indicated lightness/ brightness, redness/greenness and yellowness/blueness, respectively. The colorimeter was calibrated with a white standard (Olivares et al., 2010).

2.2.3. Sensory evaluation

The following Nham products; GABA Nham, controls (T_{NN} and T_{NM}) and two popular commercial Nham brands were organoleptically evaluated for color, texture, odor, flavor and sourness, and overall acceptance by 30 panelists (15 for uncooked and 15 for cooked Nhams). A five-point hedonic scale was used for sensory evaluation (Thai Industrial Standard number, 1219-2547). The scores 1, 2, 3, 4 and 5 were defined as dislike extremely, dislike, moderate acceptable (neither like nor dislike), like and like extremely, respectively. Nham samples were cut into roughly 1 cm^3 for both the uncooked and cooked specimens in a microwave for 30 sec before serving. Both uncooked and cooked Nham samples were separately served in different rooms. The samples with three digit random members were served (3 pieces/ recipe/person) on a white paper plate at room temperature. Panelists were instructed to rinse their mouths with water before starting and between each set of sample evaluations.

3. Effect of starter cultures on levels of BAs and cholesterol in Nham

3.1. Nham production

P. pentosaceus HN8 and *L. namurensis* NH2 in a ratio of 1:1 (6 log CFU/g for each strain) were used as a mixed starter culture to produce Nham and fermented for 96 h (see details on how to produce Nham and to determine the GABA content in previous section 2.1.3. To produce Nham that was enriched with GABA, 0.5% MSG was added as a substrate for GABA production and this set was called GABA Nham (T_{SM}) while another set of inoculation without addition of MSG was called T_{SN}. There were 2 control sets (spontaneous fermentation); one with no addition of starter cultures or MSG (control A, T_{NN}) and control B with addition of only 0.5% MSG

(T_{NM}). They were used for investigating the BAs and cholesterol contents including the distribution of LAB during the fermentations.

3.2. Analysis of the BAs of raw material and Nham samples

BAs were determined by HPLC according to methods described by Tosukhowong et al., (2011) and Önal et al., (2013). The following 7 BAs; tyramine, putrescine, cadaverine, histamine, β-phenylethylamine, spermidine and spermine were determined in this study due to they being the most important BAs found in foods (Shalaby, 1996), All BA standards were from Sigma Chemical Co., St. Louis, Mo., U.S.A. and separately prepared in deionized water at a concentration of 1,000 mg/L. Based on commercial Nham production, addition of MSG may be used or not used depending on their own recipes. Therefore, the amounts of BAs were investigated in Nham samples (controls: T_{NN}, T_{NM}; and inoculated sets: T_{SN}, T_{SM}). Each 5 g of Nham samples or ground pork were used to extract BAs by adding 15 mL of 0.4 M perchloric acid containing 125 µL of 1, 7-diaminoheptane as an internal standard and the mixture was homogenized for 5 min. The homogenate was centrifuged at 6,000 x*g* at 4ºC for 5 min. The supernatant was collected and the residue was extracted twice with 10 mL of 0.4 M perchloric acid solution. All supernatants were combined and the final volume was adjusted to 25 mL with 0.4 M perchloric acid. The extract was filtered with a Whatman No.1 filter paper. Adding 60 µL sodium hydroxide (2 N, NaOH), 90 µL of saturated sodium bicarbonate and 600 µL of a dansyl chloride solution (10 mg/mL into the extracted sample or standard solution (300 µL). After incubation at 40ºC for 45 min in the dark, 30 µL of 25% (v/v) ammonia solution was added to the reaction mixture for the removal of residual dansyl chloride. This reaction mixture was centrifuged for 5 min at 3,500 x *g*. The supernatant was filtered through a 0.45 µm syringe filter with a PVDF Membrane for HPLC analysis. A Zorbax Eclipse XDB C_{18}, (150 x 4.6 mm inner diameter, 5 µm) column was used with the mobile phase acetonitrile containing 0.1% (v/v) acetic acid (solvent A) and 0.1% (v/v) acetic acid (solvent B) at a flowrate of 1.5 mL/min. The column was equilibrated

with 45% solvent A and 55% solvent B for 38 min before the next injection. The column was kept at 40°C in a heated column compartment. A 20 µL sample was injected, and the dansylated amines were detected at 254 nm with 550 nm as the reference.

3.3. Analysis of Cholesterol in Nham samples

To analyze for the cholesterol content in Nham, there was only control A (T_{NN}) and GABA Nham, T_{SM}) at the beginning and the end of fermentation to determine if the amount of MSG had any effect on the cholesterol content. The cholesterol content in the most popular commercial Nham brands (A and B) was also determined for making a comparison. The method of Moses et al., (1975) was adopted with some modification as follows. Nham samples were weighed (1 g for each sample) and placed into a 125 mL boiling flask, followed by 250 mg of pyrogallol and 50 mL of 95% ethanol and then 25 mL of 60% potassium hydroxide (KOH). The mixture was stirred, boiled, and refluxed for 30 min, as compared to 75-80°C in the original procedure followed by cooling at 25°C. A 1 min mixing was required before the solution was transferred to a 250 mL separating funnel, 50 mL of hexane and 25 mL of distilled water were added into each separating funnel. At least two washes of hexane extract were used to eliminate aqueous components. After incubation at room temperature for 5 min to allow phase separation, the final hexane layer was transferred into a clean flask containing anhydrous sodium sulfate; so that all the moisture associated with hexane was removed. The hexane layer was collected and evaporated at 40°C. The stock reagent was prepared by dissolving 0.1 g of $FeCl_3.6H_2O$ in 5 mL of deionized water and 95 mL of glacial acetic acid using a 100 mL volumetric flask. Prior to use, 86 mL of concentrated H_2SO_4 was added. The stock solution (2 mg/mL) was prepared by dissolving 200 mg of standard cholesterol (chromatography grade; Sigma Chemical, USA) in 1-propanol using a 100 mL volumetric flask. The stock solution was used to prepare working solutions containing 0.05, 0.10, 0.15 0.20 and 0.25 mg cholesterol/mL. A 3 mL of the $FeCl_3$ coloring

reagent was added into a total volume of each working solution (1 mL), and the developed color was measured at 540 nm using a spectrophotometer. A standard curve was built by using the standard concentration of cholesterol for the abscissa and the absorbance of each working solution for the ordinate.

4. Succession of LAB during Nham fermentations

4.1. Nham samples

Samples during Nham fermentations (controls: T_{NN}, T_{NM}; inoculated sets: T_{SN}, T_{SM}) were use to count and isolate LAB at 0, 24, 48, 72, 96 h with the aim to monitor the LAB succession. This was done by mixing 25 g of each Nham sample thoroughly with 225 mL of sterile 0.85% NaCl to give 1:10 dilution and a 10^{-6} dilution was made. The pour plate technique was used with the appropriate dilutions in MRS agar plates and incubated at 30^{o}C for 24 h. Representative colonies (RC) were selected at random based on their different morphologies, and the numbers of isolates was calculated as a square root of the colonies of the LAB found in the appropriate MRS plate (Harrigan and McCance, 1976).

4.2. Conventional method

The RC isolates were purified followed by a catalase check, and pure catalase negative isolates were examined for cell shape, cell arrangement, and their physiological and biochemical properties (Schleifer, 2009). The following properties were tested; gas production from glucose, and growth with various conditions such as temperatures of 15/45ºC; pH 4.5/8.5; and 5, 6, 7 and 10% NaCl; including 16 carbohydrate fermentation tests. One percent of each carbohydrate was tested:

amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, raffinose, rhamnose, ribose, sorbitol, sucrose and trehalose. In addition, a 10% of representative colonies were also identified using 16S rDNA sequence analysis (see details 1.4) to confirm the results of the conventional method.

4.3. DGGE analysis of Nham samples

4.3.1. Direct extraction of total DNA from Nham

Total DNA extraction of Nham samples and pure cultures from subsection 4.1 was performed using a commercial DNA isolation kit (QIAamp DNA Mini kit, Qiagen, Germany) according to the manufacturer's protocol for Gram-positive bacteria. A representative 20 g of each Nham sample was homogenized in 20 mL of saline-peptone water for 3 min in a stomacher. Two mL of homogenized Nham suspension and 2 mL of each overnight bacterial culture were separately conducted for total DNA extraction. All suspensions were centrifuged at 10,000 *xg* for 10 min and each pellet were then resuspended in 180 μL of lysozyme solution (20 mg/mL lysozyme, 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton X-100) (Kesmen et al., 2012).

4.3.2. PCR amplification for DGGE

The genomic DNA of LAB isolated from Nham and Nham samples were used for amplification of 16S rRNA gene. The universal 16S rDNA primers, forward primer 8F (5’-GAGTTTGATCCTGGCTCAG-3’), and reverse primer 1492R (5’-CGGYTACCTTGTTACGACTT-3’) were used for the polymerase chain reaction (PCR). The PCR amplification was carried out in a reaction mixture containing ~100

ng genomic DNA as template, 10 µL of 2x iProof High-Fidelity Master Mix (contain 1.5 mM $MgCl_2$, 200 µM dNTP each) (Bio-Rad), 0.5 pmol/mL each of forward and reverse primer, and ddH_2O enough to make up the volume to 20 µL reaction mixture. Amplification conditions were 30 s initial denaturation at 98^oC, 10 s denaturation at 98^oC, 30 s of primer annealing at 56^oC, 30 s elongation at 72^oC for 35 cycles; and a final extension of 10 min at 72^oC. The reactions were carried out in a Thermal Cycler (IQ Bio-Rad).

An internal 177 bp fragment of the V3 region of the 16S rRNA gene was amplified with the universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). A GC-clamp (5'-CCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3') (Temmerman et al., 2004) was coupled to the forward primer 341F to improve DGGE separation (Myers et al., 1989). Amplification reactions were carried out in a 50 µL reaction volume containing 25 µL 2x iProof High-Fidelity Master Mix, 1.25 µL each primer (5 pmol/mL), 2.5 µL DNA template, and 20 µL ddH_2O. The samples were amplified in a Thermocycler with the program as follows: after pre-incubation at 95°C for 5 min, a total of 35 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min and finally 72°C for 7 min and cooling to 4°C. The PCR products (5 µL) were analyzed by electrophoresis on an agarose gel (1.5%) at 100 V for 45 min to check the sizes and amounts of the amplicons.

4.3.3. DGGE analysis

PCR products of V3 region of 16S rDNA were analyzed on DGGE gels based on the protocol of Muyzer et al., (1993). Polyacrylamide gels (160×160×1 mm) consisted of 8% (v/v) polyacrylamide (Bio-Rad, Hercules, USA) in 1×TAE buffer (Bio-Rad) and a 35% to 55% denaturing gradient (100% denaturing polyacrylamide solution, corresponding to 7 M urea, and 40% (w/v) formamide. Electrophoresis was performed for 10 min at 200 V, followed by 16 h at 85 V in 1×TAE buffer at a

constant temperature of 60°C using the Dcode System (Bio-Rad, Eke, Belgium). Gels were stained with SYBR Gold (solution of 50 μL in 500 mL 1×TAE buffer) for 30 min, followed by visualization and digital capturing using the Molecular Imager Gel Doc XR System (Bio-Rad).

4.3.4. Sequencing of DGGE bands

Bands of interest were excised from acrylamide gels by inserting a pipette tip into the band and the DNA was left to elute overnight at 4°C in 30 μL 1×TE buffer. Before sequencing, the position of each excised band was compared to its relative position in the original DGGE profile. The extracted DNA was reamplified using the same protocol as described above for DGGE. Primers 341F (without GC-clamp) and 518R were used for sequencing. The BLAST algorithm was used to determine the most similar sequences in the EMBL nucleotide sequence database. The band was identified based on the highest similar sequences of the V3 region 16S rRNA gene of one species in the National Center for Biotechnology Information databases (NCBI). A tentative identification was based on those entries exhibiting the highest percentage of similarity as requiring a minimum level of 97% similarity.

5. Statistical analysis

Statistical analysis was performed using a multivariate of Tukey's B test when $P < 0.05$ indicates significant differences in GABA production, culture filtrate pH and antibacterial activity. Probiotic properties and other experiments were analyzed using a one-way ANOVA with post hoc Duncan's test when $P < 0.05$ is considered as significant differences. A T-Test was used for comparison of pairs. Analysis was performed using the SPSS package (SPSS 16.0 for windows, SPSS Inc, Chicago, IL,

USA). All data are expressed as a mean of three determinations ± its standard deviation.

CHAPTER 4
RESULTS AND DISCUSSION

1. Isolation and selection of LAB to be used as starter cultures

1. 1. Isolation of LAB

Among 163 samples of fermented foods, LAB strains were detected in 162 samples (99.4%). No LAB strains were found in one sample of Plara. After consideration of distinctive colonies, 602 LAB were isolated (Table 4.1). Results showed that Nham (fermented ground pork) was the most enriched source of LAB (6 isolates/sample), followed by Fish Nham (fermented ground fish) (roughly 6 isolates/sample) and Pork Nhang (fermented sliced beef) (roughly 4 isolates/sample). As NaCl was not added to the MRS medium the presence of no LAB in one Plara sample might be due to the high content of salt (NaCl) in this product when compared with other fermented food products used in this study.

Table. 4.1. Isolation of lactic acid bacteria from various Thai fermented foods.

Fermented foods	Thai names of the foods	No. of samples	No. of detected samples	No. of isolates
Fermented ground pork	Pork Nham	54	54	320
Fermented fish	Fish Nham	6	6	33
Fermented sliced beef	Beef Nhang	22	22	55
Fermented sliced pork	Pork Nhang	12	12	45
Fermented shrimp	Kung-Som	28	28	45
Fermented whole fish	Pla-Som	18	18	55
Fermented red fish	Plapangdang	17	17	35
Fermented sour sausage	Isan sausage	5	5	12
Fermented fish paste	Plara	2	1	2
Total		164	163	602

1.2. Screening of GABA producing LAB

Table 4.2 shows that all 602 LAB isolates grew in MRS broth under static condition over 18 h period of incubation with different growth rates. There were 4 levels of bacterial growth as follows: excellent, very good, good, and fair and the percentage in each level was 8, 38, 43 and 11% respectively. A total of 276 LAB isolates that were ranked in the groups of excellent and very good growth were further investigated for their production of GABA. 149 isolates produced GABA in the MRS broth containing 0.5% MSG (Table 4.3) as shown by detection of GABA using TLC (Figure 4.1). The R_f values of MSG and GABA were 0.26, and 0.36, respectively.

Roughly 54% of the tested LAB produced GABA and secreted this compound into the medium. This means that extracellular GABA production was observed in LAB culture broth and it is possible to use the GABA producing LAB as starter cultures in fermented foods. Therefore, all GABA producing LAB strains were further selected based on the amount of GABA they produced and strains with the highest amount of GABA will be used as starter cultures.

Table. 4.2. Growth of LAB isolated from fermented foods in MRS broth after 18 h cultivation under static condition.

Growth (OD_{660} nm)	Growth level	Number of isolates	Percent of isolates
> 1.5	Excellent	49	8.14
> 1.0-1.5	Very good	227	37.71
0.5-1.0	Good	259	43.02
< 0.5	Fair	67	11.13
Total		602	100

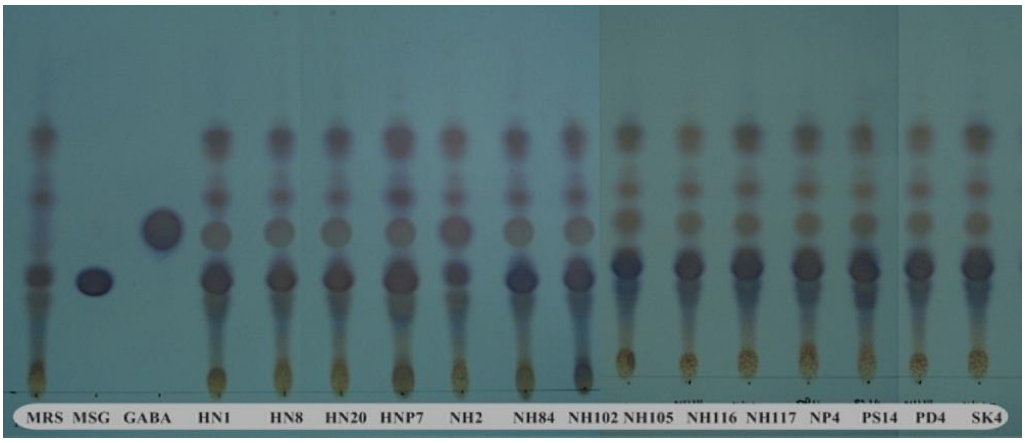


Figure 4.1. Thin layer chromatography chromatogram of GABA production by isolated lactic acid bacteria. Lanes: 1, MRS broth; 2, 0.1 M monosodium glutamate (MSG) standard; 3, 0.1 M γ-aminobutyric acid (GABA) standard: 4–17, lactic acid bacteria tested.

Table. 4.3. Lactic acid bacteria isolated from Thai fermented foods that produced GABA

Fermented foods	Thai name	Number of isolates tested	GABA producing LAB (% of isolates)*
Fermented ground pork	Pork Nham	154	75 (48.7)
Fermented fish	Fish Nham	8	5 (62.5)
Fermented sliced beef	Beef Nhang	26	16 (61.5)
Fermented sliced pork	Pork Nhang	15	11 (73.3)
Fermented shrimp	Kung-Som	18	16 (88.9)
Fermented whole fish	Pla-Som	30	21 (70.0)
Fermented red fish	Plapangdang	19	4 (21.1)
Fermented sour sausage	Isan sausage	4	1 (25.0)
Fermented fish paste	Plara	2	- (0)
Total		276	149

* Number in a bracket is the percentage of the isolates that produced GABA

1.3. Selection of GABA-producing LAB and their antibacterial activity

Among 149 GABA-producing LAB, only 14 isolates clearly produced GABA based on the TLC results (Figure 4.1); these 14 strains were further investigated for measuring the amount of GABA using HPLC. Table 4.4 shows the amount after 24 h cultivation in MRS broth supplement with 0.5% MSG. Strain HN8 was the best GABA producer (9,060 mg/L) followed by strains NH116 (8,411 mg/L), NH102 (8,386 mg/L) and NH117 (8,327 mg/L). These were selected for further studies. There were 8 strains that produced GABA more than 6,000 mg/L, but <8,000 mg/L (NP4, HNP7, NH2, PS14, PD4, NH105, HN1 and SK4). A few strains produced GABA <6,000 mg/Land the least was strain NH84 (3,506 mg/L). Over 48 h

in MRS broth, all GABA-producing LAB strains produced pH values in a narrow range (3.8–4.2) and their inhibitory spectra of their culture filtrates are presented in Table 4.4. Most of them inhibited the growth of all target organisms. *C. perfringens* DMST16637 was the most tolerant strain (inhibition zone <10–15 mm), while *L. monocytogenes* DMST 4553 and *S. sonnei* PSSCMI 0034 were the most sensitive strain (inhibition zone > 15–25 mm).

According to Table 4.4, the following strains; HN8, NH102, HN1, HN20 and NH84 gave the most inhibition against both the spoilage organism (*E. coli* ATCC25922) and the pathogenic bacteria. In contrast, the following strains; HNP7, NH105 and SK4 produced the lowest inhibition against all the target organisms because some did not inhibit the growth of *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *E. coli* ATCC 25922 and *S. Typhi*. In addition, the worst group did not inhibit the growth of *L. monocytogenes* DMST4553, although all of them gave a fair growth inhibition to *S. sonneii* and *C. perfringens* DMST16637. An interesting result was observed for strain NH2, as its culture filtrate at pH 4.2 strongly inhibited the serious pathogens (*S. aureus* and *S. Typhi*.) found in Nham. The best strain for producing GABA and antibacterial activity against the growth of pathogens, including the spoilage organism, was HN8 followed by strains NH102 and NH116. However, strain NH2 was graded in the second best group, as it had high antibacterial activity at a higher pH (4.2) with 0.90% total acidity compared to other strains (pH 3.8, 1.44– 1.49% total acidity), and also produced a high amount of GABA. Hence, this might help to reduce post acidification of Nham due to a lower acidity after 4 fermenting days. In general, the best taste of Nham product occurs between 3 days and 4 days of lactic fermentation, as the mixed ingredients of ground pork, sliced skin pork, garlic, chilli, salt and a small amount of potassium nitrate obtain a sour taste with a good texture. In addition, strain NH102 inhibited bacterial pathogens to the same level as strain HN8, whereas strain NH 116 produced almost as much GABA as strain HN8. According to above results the following strains; HN8, NH2, NH102 and NH116 were selected for investigating their potential as probiotics.

Table 4.4. Properties of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB), including inhibitory action against food-borne pathogenic bacteria and spoilage organisms

LAB strains	Source of LAB (fermented foods)	GABA (mg/L)*	pH†	Degree of inhibition ‡						
				Bacillus cereus	*Clostridium perfringens*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella* Typhi	*Shigella sonneii*
HN8[A]	Sliced beef	9060[a]	3.8	++	+	++	++	++	++	++
NH116[B]	Ground pork	8411[b]	3.8	++	+	++	+	+	+	++
NH102[A]	Ground pork	8386[c]	3.8	++	+	++	++	++	++	++
NH117[B]	Ground pork	8327[c]	3.8	+	+	++	+	+	+	++
NP4[C]	Ground fish	7493[d]	3.9	+	+	+	+	+	+	+
NH2[B]	Ground pork	7339[f]	4.2	+	+	+	++	+	++	+
PS14[B]	Whole fish	7154[g]	4.0	++	+	++	+	+	+	++
PD4[B]	Red fish	6973[h]	3.9	++	+	++	+	+	+	++
HN1[A]	Sliced beef	6785[i]	3.9	++	+	++	++	++	++	++
HN20[A]	Sliced beef	5569[k]	3.8	++	+	+++	++	++	+	+
NH84[A]	Ground pork	3507[l]	3.8	++	+	++	++	++	++	++
HNP7[D]	Sliced pork	7389[e]	4.0	+	+	-	+	-	-	+
NH105[D]	Ground pork	6816[i]	3.8	+	+	-	-	+	-	+
SK4[D]	Sour sausage	6381[j]	3.9	-	+	-	-	+	+	+

Mean values with different lowercase letters in the same column (GABA) and uppercase letters in the LAB strains column (antimicrobial activity) indicate significant differences ($P < 0.05$). The degrees of inhibition were set as 1, 2 and 3 for +, ++ and +++, and then the degree of inhibition on all target organisms for each LAB strain was summed for statistical analysis.

*Produced in MRS broth plus 0.5% MSG;

†Culture filtrate pH (no significant differences);

‡The inhibition was graded by the radius of the inhibition zone using the following scales: no inhibition = - , < 10-15 mm = + (fair), > 15-20 mm = ++ (good), >20-25 mm = +++ (very good).

Based on the amount of GABA produced and their antibacterial activity *P. pentosaceus* HN8 isolated from a sliced fermented beef was the best lactic acid bacterium for use as a starter culture followed by strains NH102 and NH116 that were isolated from a fermented ground pork samples (Table 4.4 and Figure 4.1). However, *L. namurensis* NH2 that originated from fermented ground pork also produced high amounts of GABA and had antibacterial activity at a higher pH than other strains. As pediococci are normally most prominent during the early stages of fermentation (Ammor et al., 2005; Ho et al., 2009), in this study *P. pentosaceus* strains were isolated from the finished products of fermented meats. Therefore, they have the potential to be used as probiotics in fermented meats; and this is the first to report that *P. pentosaceus* is a good producer of GABA. In general, *Lactobacillus* and *Lactococcus* spp have been previously reported to produce GABA (Park and Oh, 2007; Kim et al., 2009; Ratanaburee et al., 2011a). We are the first to report that *L. namurensis* was also a good GABA producer. *L. namurensis* was recently considered to be a novel species of *Lactobacillus* isolated from a traditional Belgian sourdough (Scheirlinck et al., 2007). Strain NH2 originated from a ground pork fermentation but cooked rice could also have been its source as they are main ingredient for making Nham.

Antibacterial activity against potential pathogens and spoilage organisms is an attractive property for use as a starter culture because it could help to achieve safe fermented foods (Prachyakij et al., 2008; Kantachote et al., 2010). Amongst the GABA producing LAB strains tested, their final pH values were between 3.8-4.2; however, they all inhibited target organisms. This indicated that pH and organic acids might not be the only source of antibacterial activity in their culture filtrates, especially for strain NH2 (pH 4.2) that had a high inhibitory effect against the growth of pathogens (Table 4.4). In addition, a culture filtrate at pH 6.5 from this strain also inhibited pathogens (data not shown). Hence, it would be interesting to use this strain as one of starter cultures as it could help to reduce post-acidification of fermented ground pork.

1.4. Identification of GABA-producing LAB

The biochemical and physiological properties of selected LAB strains are shown in Table 4.5. Strain HN8 was isolated from a fermented sliced beef product whereas strains NH102 and NH116 were isolated from fermented ground pork samples. These strains were spherical, catalase negative; and no gas production from glucose (homofermentative) was observed. They grew in the presence of 5, 6 and 7% NaCl, 15-45ºC and at pH 4.5-8.5. Based on the results, they were classified as *Pediococcus* (Garvie, 1986). Strain HN8 utilized the following carbohydrates; amygdalin, cellobiose, esculin, fructose, galactose, glucose, lactose, mannitol, ribose, sorbitol and sucrose (see details in Table 4.5 for strains NH102 and NH116). With some different results for consuming carbohydrate; however, results of 16S rDNA sequence analysis proved that all of them were *Pediococcus pentosaceus* with 100% similarity (Figure 4.2). Strain NH2 was isolated from a fermented ground pork had rod shaped cells, was catalase negative and produced gas from glucose (heterofermentative). It grew at 15ºC but not for 45ºC, pH 4.5; but it had poor growth at pH 8.5 and 10% NaCl. Among 16 carbohydrates tested, only glucose, maltose, mannitol, and ribose were consumed by this strain.

The partial sequences of 16S rDNA of 4 LAB strains; NH2, HN8, NH102 and NH116 were compared with other bacterial strains in the GenBank database. The result showed that LAB strains HN8, NH102 and NH116 were 100% similarity to *P. pentosaceus* while strain NH2 was 100% identity to *L. namurensis*. These data were supported by phylogenetic tree that was reconstructed with other strains of closely related LAB available from the GenBank. It was found that strains HN8, NH102 and NH116 were formed a monophyletic group with a strain of *P. pentosaceus* strain LAB2 (JN039348) while strain NH2 was formed a monophyletic group with *L. namurensis* strain Ln-15 (HM130541). It could be concluded form phenotypic and molecular characteristics that our strains HN8, NH102 and NH116 were *P. pentosaceus* and strain NH2 was *L. namurensis*.

Table 4.5. Biochemical and physiological properties of selected γ-aminobutyric acid (GABA)-producing lactic acid bacteria isolated from fermented meat products.

	Strain					Strain			
Property	**HN8**	**NH102**	**NH116**	**NH2**	**Property**	**HN8**	**NH102**	**NH116**	**NH2**
Shape	Sphere	Sphere	Sphere	Rod	5. Fructose	+	+	+	–
Gram stain	+	+	+	+	6. Galactose	+	+	+	–
Catalase test	–	–	–	–	7. Glucose	+	+	+	+
Gas from glucose	–	–	–	+	8. Lactose	+	–	–	–
Growth at 15/45 °C	+/+	+/+	+/+	+/–	9. Maltose	–	–	+	+
Growth at pH 4.5/8.5	+/+	+/+	+/+	+/±	10. Mannitol	+	–	–	+
Growth at 5%, 6% and 7% NaCl	+	+	+	+	11. Raffinose	–	–	–	–
10% NaCl	±	+	–	±	12. Rhamnose	–	–	–	–
Carbohydrate fermentation									
1. Amygdalin	+	–	–	–	13. Ribose	+	+	+	+
2. Arabinose	–	–	–	–	14. Sorbitol	+	–	–	–
3. Cellobiose	+	+	+	–	15. Sucrose	+	+	+	–
4. Esculin	+	+	+	–	16. Trehalose	–	–	–	–

+, positive reaction; –, negative reaction; ±, delayed reaction.

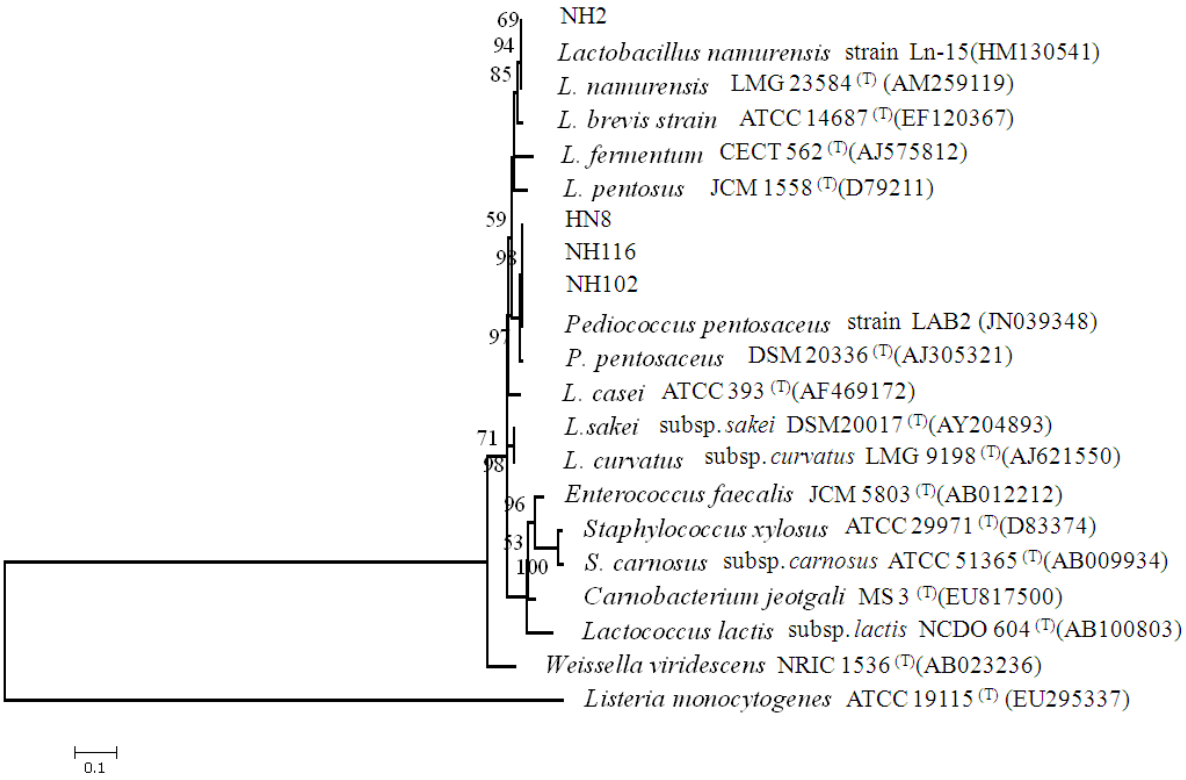


Figure 4.2. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences. The relationships between strain HN8, NH2, NH102, NH116 and related species are shown. *Listeria monocytogenes* ATCC 19115$^{(T)}$ (EU295337) was used as an outgroup. Bootstrap values based upon 1000 replicates are included at the major branch points. Bar, 0.1 nucleotide substitutions per site.

It has long been recognized that some LAB such as pediococci and lactobacilli found in fermented foods produce antimicrobial substances such as hydrogen peroxide, diacetyl, short chain fatty acids and bacteriocins (Cleveland et al., 2001; Albano et al., 2007; Kantachote et al., 2010). Among LAB strains tested, *P. pentosaceus* HN8 and NH102 were the most effective strains to inhibit all target organisms. Results in this study were similar to previous studies that showed LAB inhibited the tested pathogens like *B. cereus*, *L. monocytogenes*, *C. perfringens* and *S. aureus* (Cleveland et al., 2001; Noonpakdee et al., 2003). However, an ability to inhibit *C. perfringens* DMST16637 was low and this may be because this pathogen is very resistant. This was in agreement with Noonpakdee et al., (2003) who found that *Lactococus lactis* WNC20 isolated from Nham produced a poor inhibition on *C. perfringens* 11147. Hence, it is possible that LAB isolated from Nham can not control *C. perfringens*. *C. perfringens* is an anaerobic, spore forming, bacillus; therefore it should be further studied because it may be detected when Nham is being made with unhygienic conditions. It was of interest that, *P. pentosaceus* HN8, NH102 and *L. namurensis* NH2 strongly inhibited both serious pathogens (*S. aureus* and *S. typhi*) that are frequent contaminants in Nham.

1.5. Probiotic properties of selected LAB *in vitro*

Table 4.6 shows results of the probiotic properties of 4 selected LAB strains. All 4 LAB strains with their initial cell density in a range of 5.5×10^8 - 1.2×10^9 CFU/mL survived in the simulated stomach condition for 2 h by between 4.0×10^8 - 9.0×10^8 CFU/mL, whereas their survival at 4 h sequential exposure to simulated intestinal juice after incubating in simulated gastric juice was between 3.3×10^7- 1.1×10^8 CFU/mL. A remarkable decrease of LAB strains was found after 4 and 6 h exposure although there was no significant difference among strains tested both in the simulated gastric juice (2 h) and intestinal juice after 2 h exposure. They showed different degrees of resistance on the biological barrier test and the best resistant

strain was NH102. There was no significant difference in the growth of all strains tested under aerobic and anaerobic conditions as indicating by the ORP values.

Results of the hydrophobicity test using hexadecane droplets revealed that the strain HN8 produced the highest value of 11.57%, followed by 8.29, 5.75 and 1.59% in strains NH2, NH102 and NH116, respectively. No α or β blood haemolysis was observed for any tested isolate. All strains of *P. pentosaceus* (HN8, NH102 and NH116) gave the same result of BSH activity on plates as they all deconjugated GC, TC, GDC and TDC. On the contrary, *L. namurensis* NH2 gave a poor result to deconjugate GC, TC and a negative result for GDC and TDC. Amongst the strains tested, NH116 was the best strain to reduce cholesterol (43.32%) followed by strain HN8 (36.93%) and the lowest was found by strain NH2 (31.25%). In addition, key results from Table 4.4 (GABA production, antibacterial activity, pH and total acidity) were included in Table 4.6 as some properties are considered as probiotic properties; and this might help to make a decision for suggesting recommended strains to be used as starter cultures.

Susceptibility of the 4 tested LAB strains to 15 commonly used antibiotics is presented in Table 4.7 With regard to the disc diffusion testing established by the National Committee for Clinical Laboratory Standards (NCCLS), which is now known as the Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 2004; Stephen et al., 2005), amongst the LAB tested, *P. pentosaceus* was considered more sensitive to antibiotics than *L. namurensis*. All strains of *P. pentosaceus* tested were sensitive to the following antibiotics: cefoperazone, cephalothin, chloramphenicol, erythromycin, and penicillin G, except for strain HN8 that was not sensitive to cefoperazone. On the other hand, strain NH2 was the most resistant to 8 antibiotics (bacitracin, ceftazidime, gentamycin, kanamycin, norfloxacin, polymyxin B, streptomycin and vancomycin). Two antibiotics (ampicillin and tetracycline) inhibited all strains tested at an intermediate level.

All of the selected LAB strains in this study could survive in the presence of bile salts and pancreatin conditions, even with pre-exposure of stomach condition at pH 2.0 for 2 h (Table 4.6). One of the reasons to explain why tested LAB strains

could survive in such conditions is due to their activity to hydrolyze various bile salts (GC, TC, GDC and TDC) via BSH so decreasing solubility of bile salts. It is well recognized that undissociated form of bile salts can acts as antimicrobial agent like organic acids; thereby cell membrane of microbes is altered by bile salts (Pennacchia et al., 2004). Based on above results suggest that all LAB strains tested might be able to compete in the human intestine. In addition of BSH activity, the selected LAB strains also showed ability to reduce cholesterol from MRS broth and a higher BSH activity was found in all tested strains of *P. pentosaceus*.

Probiotic bacteria generally colonize at the small intestine with strictly anaerobic conditions, thereby probiotic bacteria at least they should be facultative anaerobe. Fortunately, all of selected LAB strains were facultative anaerobe (Table 4.6); and the results were in accordance with previous studies (Kantachote et al., 2010). The adhesion of microorganisms to intestinal epithelial cells can be considered to be the first step of colonization. Hence, cell hydrophobicity may contribute to adhesion of bacterial cells to host tissues; and this property could indicate an advantage and importance for bacterial maintenance in the human GI tract (Ram and Chander, 2003). All selected LAB strains gave a low percentage of cell hydrophobicity with hexadecane droplets; however, it was in agreement with our previous work (Kantachote et al., 2010) that *L. plantarum* DW3 and *L. casei* R (commercial strain) also gave the same level of cell hydrophobicity (roughly 11-12%). The strain DW3 was proven to act as a probiotic bacterium in ICR mice although it had a low percentage of cell hydrophobicity (Kantachote et al., 2010).

The antibiotic susceptibility of selected LAB strains test was investigated because is the one of criteria safety evaluations in probiotics. Due to probiotics are used with living organisms; therefore if they have antibiotic resistance genes and transferring them to pathogenic bacteria may be risk for use. On the other hand, recently many researchers have proposed that co-administration of probiotics and antibiotic associated diarrhea patients might help to change the balance of normal flora in GI tract to become normal (Hickson et al., 2007). Results in this study (Table 4.7) was in accordance with previous studies (Gevers et al., 2003; Kantachote et al., 2010; Yuksekdag and Aslim, 2010) as starter LAB used in sausage and beverage

normally resisted to the following antibiotics; bacitracin, gentamycin, kanamycin, norfloxacin, polymyxin B, streptomycin and vancomycin. Regarding to haemolytic activity, all selected LAB strains in this study clearly showed no this activity and this is a sign to ensure the use of these organisms as starter cultures for Nham fermentation.

Overall these results clearly show that the ability to produce large amounts of GABA, inhibit potential pathogens and be effective as a probiotic starter is independent of genera and source. Each isolate has its own unique characteristics. Among 4 strains (HN8, NH102, NH116 and NH2) were potential candidates to be used as starter cultures. However, strain HN8 was the best strain to produce GABA and antibacterial activity while strain NH2 produced less acid than most other selected LAB and so reduce post acidification. Hence, strains HN8 and NH2 are suitable to be used as starter cultures to produce a novel Nham product; thereby they were further tested for their ability to act as starter cultures for Nham production.

Table 4.6. Probiotic properties *in vitro* testing of selected γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) strains for using as starter cultures to produce Nham.

LAB strains	***Pediococcus pentosaceus* HN8**	***Pediococcus pentosaceus* NH102**	***Pediococcus pentosaceus* NH116**	***Lactobacillus namurensis* NH2**
Source	**Fermented sliced beef**	**Fermented ground pork**	**Fermented ground pork**	**Fermented ground pork**
Survival (CFU/mL)				
Control (0 h)	$9.5 \times 10^{8\ a}$	$1.2 \times 10^{9\ a}$	$1.2 \times 10^{9\ a}$	$5.5 \times 10^{8\ a}$
Simulated gastric juice (pH 2.0) 2 h	$7.0 \times 10^{8\ a}$	$9.0 \times 10^{8\ a}$	$9.0 \times 10^{8\ a}$	$4.0 \times 10^{8\ a}$
Simulated intestinal juice (pH 8.0) 2 h	$4.2 \times 10^{8\ a}$	$5.4 \times 10^{8\ a}$	$5.0 \times 10^{8\ a}$	$2.5 \times 10^{8\ a}$
4 h	$3.8 \times 10^{7\ c}$	$1.1 \times 10^{8\ a}$	$7.3 \times 10^{7\ b}$	$3.3 \times 10^{7\ c}$
6 h	$2.0 \times 10^{5\ c}$	$3.2 \times 10^{6\ a}$	$5.5 \times 10^{5\ b}$	$6.5 \times 10^{5\ b}$
Viable cells (CFU/mL)				
Aerobic conditions (ORP, mV)	$1.00 \times 10^{9\ a,A}$ $(41.00)^{a}$	$9.33 \times 10^{8\ a,A}$ $(19.00)^{b}$	$9.00 \times 10^{8\ a,A}$ $(13.50)^{c}$	$1.77 \times 10^{8\ b,A}$ $(44.00)^{a}$
Anaerobic conditions (ORP, mV)	$1.86 \times 10^{9\ a,A}$ $(- 30.00)^{b}$	$6.67 \times 10^{8\ b,A}$ $(- 33.50)^{ab}$	$6.33 \times 10^{8\ b,A}$ $(- 36.00)^{a}$	$1.00 \times 10^{8\ c,A}$ $(- 34.50)^{a}$
Hydrophobicity (%)	11.57 ± 0.68^{a}	5.75 ± 0.17^{c}	1.59 ± 0.16^{d}	8.29 ± 0.22^{b}
Haemolysis	γ	γ	γ	γ
Bile salt hydrolase activity*				
Glycocholic acid (GC)	+	+	+	±
Taurocholic acid (TC)	+	+	+	±
Glycodeoxycholic acid (GDC)	+	+	+	-
Taurodeoxycholic acid (TDC)	+	+	+	-
Removal of cholesterol (%)	36.93 ± 2.01^{b}	33.38 ± 1.00^{c}	43.32 ± 1.01^{a}	31.25 ± 0.00^{c}
GABA production†	9060^{a}	8386^{c}	8411^{b}	7339^{d}
Antibacterial activity†	Very good	Very good	Good	Good
Culture filtrate pH (total acidity)†	3.8 (1.49%)	3.8 (1.44%)	3.8 (1.44%)	4.2 (0.90%)
Recommended strain[2]	√			√

Mean values with different lowercase letters in the same row indicate significant differences ($P < 0.05$). Mean values with different uppercase letters in the same column indicate significant differences ($P < 0.05$),

*+ = precipitated bile acid around colonies.

†Results from Table 4.4

Table 4.7. Susceptibility of selected γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) strains to antibiotics used

LAB strains / **Antibiotic**	***Pediococcus pentosaceus* HN8 (inhibition zone, mm)**	***P. pentosaceus* NH102 (inhibition zone, mm)**	***P. pentosaceus* NH116 (inhibition zone, mm)**	***Lactobacillus namurensis* NH2 (inhibition zone, mm)**
Ampicillin (10 μg)	I (20)	I (20)	I (20)	I (20)
Bacitracin (10 μg)	I (17)	I (17)	I (12)	R (12)
Cefoperazone (75 μg)	I (20)	S (27)	S (24)	S (24)
Ceftazidime (30 μg)	R (12)	R (14)	I (16)	R (16)
Cephalothin (30 μg)	S (30)	S (35)	S (30)	I (16)
Chloramphenicol (30 μg)	S (27)	S (30)	S (27)	S (27)
Erythromycin (15 μg)	S (25)	S (29)	S (23)	S (23)
Gentamycin (10 μg)	R (0)	R (0)	R (0)	R (0)
Kanamycin (30 μg)	R (0)	R (0)	R (0)	R (0)
Norfloxacin (10 μg)	R (0)	R (0)	R (0)	R (0)
Pencillin G (10 μg)	S (30)	S (33)	S (34)	I (25)
Polymyxin B (300 μg)	R (0)	R (0)	R (0)	R (0)
Streptomycin (10 μg)	R (0)	R (0)	R (0)	R (0)
Tetracycline (30 μg)	I (16)	I (16)	I (17)	I (17)
Vancomycin (30 μg)	R (0)	R (0)	R (0)	R (0)

According to NCCLS in therapeutic terms, susceptible means that a microorganism is inhibited by a concentration of antimicrobial agent that can be attained in body fluids following standard therapeutic doses while resistant means that a microorganism is not inhibited by the concentration of an antimicrobial agent that can be attained in body fluids following standard therapeutic doses. Intermediate means that interpretation of susceptibility results that implies clinical utility in body sites in which the drugs are physiologically concentrated (e.g. quinolones and beta-lactams in urine) or when a high dosage of a drug can be used (e.g. beta-lactams) (Stephen et al., 2005).

S = Susceptible; I = Intermediate; R = Resistant;

2. Use of starter cultures for producing Nham

2.1. Optimization of the key factors to produce GABA Nham

The experimental design by CCD was used to investigate the effects of three independent variables (starter cultures: X_1, NaCl: X_2 and MSG: X_3) with 3 levels of each independent variable on the GABA content in Nham. Table 4.8 shows the 20 various combination sets and the corresponding GABA yields for both the actual and predicted values. The experimental results were analyzed using regression analysis that consisted of the effect of linear, quadratic and an interaction that provided the following regression equation with the GABA content as a function of the three independent variables.

$$Y_{GABA} = 1628.01 - 447.01X_1 + 821.00X_3 - 428.60\ X_1X_3 - 370.56\ X_2X_3 \qquad \text{(Eq. 2)}$$

A regression model for which the coefficient of variation (R^2) value was higher than 0.9; is normally considered to present a high correlation. In this case, the value of R^2 (0.9115) indicated a high correlation between the actual and predicted values from the model Equation 2. This means that this equation can be used for predicting the GABA content under conditions that varied with the three variables in the experimental range.

Among the run sets, the maximum predicted GABA content in Nham was 3,804 mg/kg in run no. 5 (Table 4.8) (X_1 = 6 log CFU/g of each, X_2 = 2.00%, and X_3 = 0.50%) while the actual value in this run was 4,051 mg/kg. Nevertheless, the maximum value of GABA content predicted from this model was 3,849 mg/kg with the optimum values of those variables; X_1-starter culture = 6 log CFU/g of each, X_2-NaCl = 2.12%, and X_3-MSG = 0.50%. After verification under optimal conditions, the GABA content in Nham was 4,000 mg/kg since the difference between the predicted and actual values was only 3.78%; thereby it should be regarded as acceptable. Consequently, the conditions of the run no. 5 (Table 4.8) produced better ‘GABA Nham ‘due to its higher GABA content than expected from the verification test.

Table 4.8. Experimental design and results of the Central Composite Design on the GABA content in Nham.

Run number	Starter (X_1)[a] (Log CFU/g)	NaCl (X_2) % (w/w)	MSG (X_3) % (w/w)	GABA content (mg/kg) Predicted	Actual
1	6.00	2.00	0.00	564	274
2	8.00	2.00	0.00	339	374
3	6.00	2.50	0.00	834	1081
4	8.00	2.50	0.00	985	569
5	6.00	2.00	0.50	3804	4051
6	8.00	2.00	0.50	1865	1450
7	6.00	2.50	0.50	2592	2389
8	8.00	2.50	0.50	1029	1150
9	6.00	2.25	0.25	1949	1503
10	8.00	2.25	0.25	1055	1284
11	7.00	2.00	0.25	1643	1560
12	7.00	2.50	0.25	1360	1109
13	7.00	2.25	0.00	681	669
14	7.00	2.25	0.50	2323	2137
15	7.00	2.25	0.25	1502	1734
16	7.00	2.25	0.25	1502	1551
17	7.00	2.25	0.25	1502	1788
18	7.00	2.25	0.25	1502	1645
19	7.00	2.25	0.25	1502	1923
20	7.00	2.25	0.25	1502	1795
R^2				0.9115	

[a] X_1 log CFU/g for each strain

2.2. Characterization of GABA Nham and fermentation

2.2.1. Microbiological changes

A similar pattern of TBC and LAB during the four Nham fermentations was found, the highest population of those two groups was detected at 24 h of fermentation and then gradually decreased until the end of the fermentation run (96 h) (Figure 4.3 A and 4.3 B). The initial population of TBC in GABA Nham (T_{SM}) (8.5 log CFU/g) was higher than the other three sets (T_{NN}, T_{NM}, T_{SN}) (6.5 log CFU/g) (Figure 4.3 A). The TBC count in GABA Nham reached a maximum number (10.2 log CFU/g) at 24 h; however, at the end of fermentation, the TBC level in all sets of Nham fermentation was in a range of 7.3-8.1 log CFU/g. In LAB, even the initial population in all sets of fermentation was approximately 6.5 CFU/g; but higher population of the two inoculation sets (T_{SN} and T_{SM}) were first detected at 24 h (9.0-9.5 log CFU/g until the end of the fermentation (7.0-8.0 log CFU/g) (Figure 4.3 B). The initial number of yeasts in all sets of Nham was roughly 6.0 log CFU/g and there was no significant change observed among them at 24 h (Figure 4.3 C). After that the yeast numbers regularly decreased and at 96 h of fermentation, the yeasts count in the GABA Nham was minimum (3.0 log CFU/g). Throughout the fermentation there was no detection of staphylococci and molds in any Nham sets (data not shown).

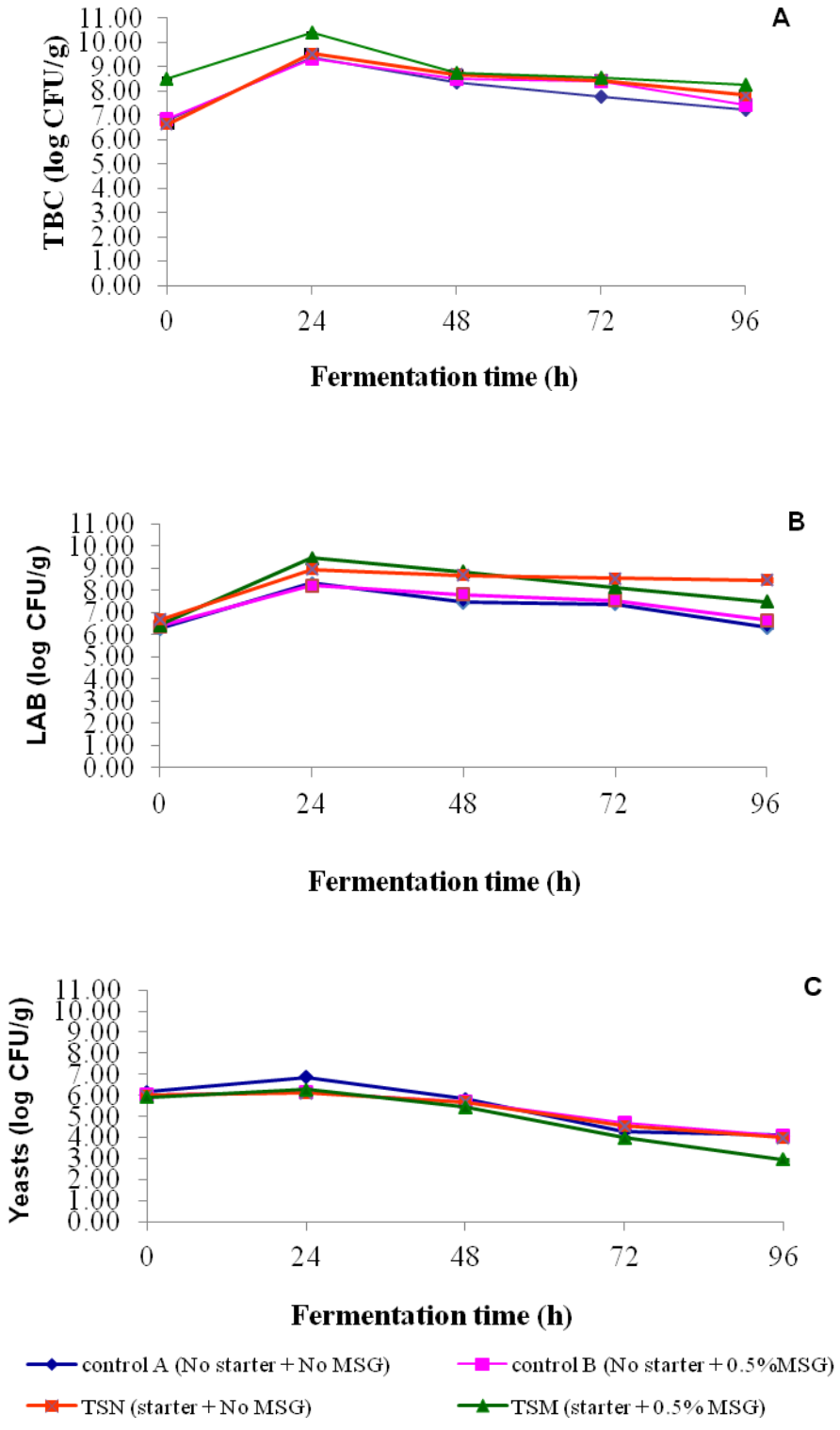


Figure 4.3. Changes of microbial populations in various Nham sets during fermentation; total bacterial count: TBC (A), lactic acid bacteria: LAB (B) and yeasts (C). Each point represents a mean of three replicates ± standard deviation.

2.2.2 Physicochemical changes

The initial pH of all sets of Nham was approximately 6.0 and the pH sharply decreased after 24 h in all fermentation sets (Figure 4.4). However, the pH value in sets with the starter cultures (T_{SN} and T_{SM}) was significantly lower than that in sets without inoculation (controls A: T_{NN}, B: T_{NM}) after 24 h and until the end of the fermentation. Thus the pH value of the inoculated sets decreased to 4.6 at 24 h; but it took 72 h in sets with no starter cultures. Nonetheless, at the end of fermentation, the final pH was approximately 4.1 and 4.3 in sets with starter cultures and no starter cultures, respectively. At the beginning all fermentation sets had a total acidity of about 0.50% but after 24 h, a remarkable increase in acidity was observed in all fermentation sets; and a significantly higher total acidity was observed in sets with inoculation. Therefore, the acidity of all finished products at 96 h obtained from starter cultures (T_{SM} and T_{SN}) was over 2.25%; whereas the acidity in sets without starter cultures was lower (controls A and B, 1.31 and 1.50%).

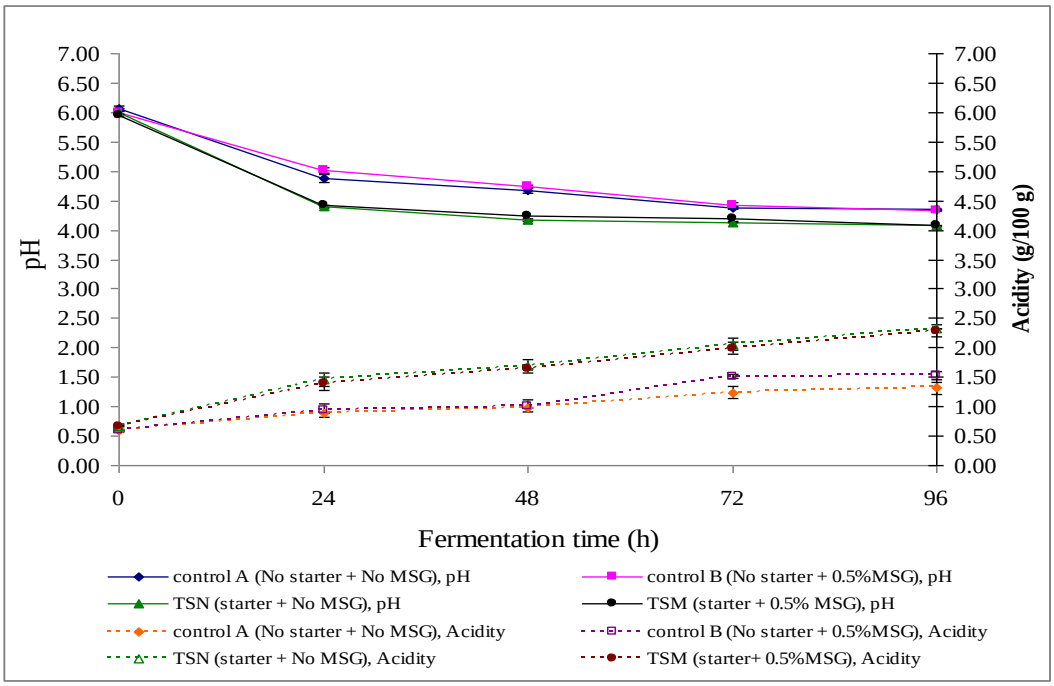


Figure 4.4. Changes of pH and total acidity in various Nham sets during fermentation. Each point represents a mean of three replicates ± standard deviation.

The maximum content of GABA, 4,051 mg/kg was found in the Nham produced with the two starter cultures (*P. pentosaceus* HN8 and *L. namurensis* NH2) and addition of MSG (Table 4.9). This concentration of GABA was substantially higher than that found in any of the commercially produced Nhams (261-792 mg/kg). Addition with MSG also stimulated the production of GABA, 828 mg/kg (control B) and lower GABA contents (220-274 mg/kg) in natural fermentation (control A) and inoculated set with no addition of MSG (Table 4.9).

Table 4.9. Effects of the starter cultures (*P. pentosaceus* HN8 and *L. namurensis* NH2 at a ratio of 1:1) and MSG additions on the GABA content in Nham by comparing with commercial Nham.

Nham recipes	GABA content (mg/kg)	SD
1. Control A (T_{NN}: No starter + No MSG)	220	3.49
2. Control B (T_{NM}: No starter + 0.5% MSG)	828	0.95
3. T_{SN} (each starter 6 log CFU/g + No MSG)	274	1.27
4. T_{SM} (each starter 6 log CFU/g +0.5% MSG)	4051	11.19
5. Commercial Nham[a]		
Nham A	320	1.63
Nham B	604	2.09
Nham C	792	10.95
Nham D	261	0.63

[a] Different starter cultures were used in some commercial Nham brands. Data are the mean values of three determinations and its standard deviation.

No significant difference was found in all sets of finished Nham products for their moisture content and ash values (Table 4.10). The finished product in the set of GABA Nham produced the least crude fat, carbohydrate and energy content at only 1.93%, 10.27% and 137 kcal when compared with other sets (2.39-2.61% for fat, 10.42-11.02% for carbohydrate and 141-146 kcal for energy). However, a higher amount of protein was observed in sets with starter cultures (19.06-20.21%) than in sets without starter cultures (18.78-20.18%). There was no significant difference in all

sets of finished Nham products for the release of water, water activity (a_w) and weight loss of Nham (Table 4.10). Values of the released water in all sets were in the range of 0.25-0.99 g/100 g whereas values of the weight loss were between 0.061 to 0.252 g/100 g.

The color and texture of the finished Nham products are presented in Table 4.11. The color of GABA Nham based on lightness (expressed as L* value, 59), redness (expressed as a* value, 8) and yellowness (expressed as b* value, 14.3) was almost similar with the remaining Nham sets although there were some significant differences from the control B (T_{NM} set) for lightness (56), the control A (T_{NN} set) for redness (8.6) and inoculated set with no addition of MSG (T_{SN} set) for yellowness (13.6). The textures of the finished Nham products were determined by comparing the results of compression hardness or firmness and toughness. The firmness and toughness of all Nham sets were significantly different ($P < 0.05$) as their values were between 45-64 N and 911-1212 N/s, respectively. The values for both firmness and toughness in a GABA Nham were 45 N and 944 N/s, respectively.

2.2.3 Sensory evaluation

Among the Nham samples tested, both uncooked and cooked GABA Nham samples had the maximum score for flavor and sourness (Table 4.12). GABA Nham also had a significant higher score than a commercial Nham A for texture, flavor and sourness including overall acceptance. Moreover, GABA Nham also was given a significantly higher score than a commercial Nham B for flavor and sourness, color appearance and overall acceptance. Overall, the results proved that GABA Nham had the highest score for all parameters tested at an average score of 4.00-4.17 out of 5 for uncooked Nham and 4.05-4.17 out of 5 for cooked Nham.

Table 4.10. Proximate compositions of Nham products.

Nham product (96 h)	Proximate analysis						
	Moisture (%)	Protein (%)	Ash (%)	Crude fat (%)	Crude fiber (%)	Total carbohydrate (%)	Energy (kcal)
Control A (T_{NN}: No starter + No MSG)	64.17^{a}	20.18^{a}	2.56^{a}	2.61^{a}	0.22^{b}	10.48^{ab}	146.13^{a}
Control B (T_{NM}: No starter + 0.5%MSG)	64.89^{a}	18.78^{b}	2.85^{a}	2.46^{a}	0.29^{a}	11.02^{a}	141.34^{c}
T_{SN} (starter + No MSG)	64.45^{a}	20.21^{a}	2.53^{a}	2.39^{a}	0.24^{b}	10.42^{ab}	144.03^{b}
T_{SM} (starter + 0.5% MSG) (GABA Nham)	65.15^{a}	19.06^{b}	2.89^{a}	1.93^{b}	0.24^{b}	10.27^{b}	137.49^{d}

Mean values with different lowercase letters in the same column indicate significant differences ($P < 0.05$), (n = 3)

Table 4.11. Changes of the physical properties in Nham products

Nham product (96 h)	Texture		a_w	Weight loss	Released	Color		
	Firmness (N)	Toughness (N/s)		(g/100 g)	water (g/100 g)	L* (lightness)	a* (redness)	b* (yellowness)
1. Control A (T_{NN}: No starter + No MSG)	47.29^{b}	910.79^{d}	0.99^{a}	0.072^{a}	0.30^{a}	59.12^{a}	8.58^{a}	13.92^{ab}
2. Control B (T_{NM}: No starter + 0.5%MSG)	63.91^{a}	1212.47^{a}	0.99^{a}	0.242^{a}	0.99^{a}	55.79^{b}	7.93^{b}	13.76^{ab}
3. T_{SN} (starter + No MSG)	45.27^{d}	937.52^{c}	0.99^{a}	0.061^{a}	0.25^{a}	58.59^{a}	8.13^{b}	13.59^{b}
4. T_{SM} (starter + 0.5% MSG)	45.42^{c}	944.13^{b}	0.99^{a}	0.252^{a}	0.97^{a}	59.02^{a}	8.02^{b}	14.29^{a}

Mean values with different lowercase letters in the same column indicate significant differences ($P < 0.05$), (n = 3)

Table 4.12. Comparison of the sensory evaluations among controls, GABA Nham and commercial Nham products

Sensory test	Control A (T_{NN}: No starter + No MSG)		Control B (T_{NM}: No starter + 0.5%MSG)		GABA Nham (starter + 0.5% MSG)		Nham A[1]		Nham B[1]	
	uncooked	cooked	uncooked	cooked	uncooked	cooked	uncooked	cooked	uncooked	cooked
Texture	3.99^{b}	4.07^{A}	4.00^{b}	4.00^{B}	4.13^{a}	4.08^{A}	3.77^{c}	3.87^{C}	4.13^{a}	4.07^{A}
Flavor and sourness	3.77^{d}	3.47^{E}	3.85^{c}	3.85^{C}	4.07^{a}	4.07^{A}	3.60^{e}	3.60^{D}	4.00^{b}	4.00^{B}
Color	4.17^{a}	4.07^{B}	4.06^{b}	4.06^{B}	4.17^{a}	4.17^{A}	4.17^{a}	4.17^{A}	3.23^{c}	3.23^{C}
Overall acceptance	3.70^{d}	3.70^{C}	3.80^{c}	3.80^{B}	4.00^{a}	4.05^{A}	3.60^{e}	3.50^{D}	3.90^{b}	3.80^{B}

Mean values with different lowercase letters in the same row indicate significant differences of uncooked Nham ($P < 0.05$).

Mean values with different uppercase letters in the same row indicate significant differences of cooked Nham ($P < 0.05$).

[1]Nham A and Nham B are popular commercial Nham products.

To our knowledge this is the first report that enhancement of the GABA content in fermented meat can occur by the use of starter cultures (*P. pentosaceus* HN8 and *L. namurensis* NH2); and the latter organism was also used for the first time as a starter culture in a fermented meat product. This study proved that both strains still have the ability to produce GABA (4,051 mg/kg) in Nham. This is in agreement with Thwe et al., (2011) who used a starter culture of GABA-producing LAB, *Lactobacillus farciminis* D323 to produce fermented tinfoil barb (N-T2), one of the traditional fermented fishery products in Myanmar eaten with boiled rice. In this case the highest GABA amount was only 2,320 mg/kg.

The numbers of LAB in the sets with inoculation after the time zero was higher than the sets without inoculation by roughly 2 log cycles (Figure 4.3B). This indicated that the starter cultures (strains HN8 and NH2) were able to adapt themselves to the normal ingredients of Nham. Normally yeasts and LAB live together in raw materials or ingredients of Nham; however, the LAB became the dominant organism within 24 h (Figure 4.3B and 4.3C). The pH dropped below 4.5 (Figure 4.4), in all sets of Nham; but GABA Nham had the minimum number of yeasts. This supported the view that in addition to organic acids the starter cultures used in this study also produced other antimicrobials compounds that could also inhibit yeasts growth. This indicated that with the use of both strains (HN8 and NH2) as starter cultures it was possible to achieve a safe Nham product.

Normally, Nham products when kept at room temperature cannot maintain their taste and texture due to the higher acidity levels with increasing time (Valyasevi and Rolle, 2002; Visessanguan et al., 2004; Rotsatchakul et al., 2009). Nevertheless, in this study one of the starter cultures, *L. namurensis* NH2 could prolong the shelf life of Nham due to the Nham product having the right consistency of sourness that was accepted by the panelists after 7 days at room temperature when compared with the controls and commercial Nham products (data not shown). Acidification and proteolysis in meat fermentation produces favorable conditions for the production of biogenic amines (BAs); however, the ability of either strain to produce BAs in Nham has not been proven in this study and that will need a further investigation. Fortunately, Bover-Cid et al., (2001) reported that no histamine producer was ever

found in fermented pork sausages. In addition, none of the LAB strains isolated from ethnic meat products from the eastern Himalayas produced BA (Rai et al., 2010).

The lowest amounts of crude fat, carbohydrate and energy in GABA Nham (Table 4.6) indicated that the starter cultures had an ability to utilize fat and carbohydrate better than the indigenous LAB. However, the microbial lipases activity in conditions of Nham fermentation are very low due to their sensitivity to low pH; it is therefore likely that lipases present in the lysosomes of the muscle tissue govern lipolysis in Nham (Visessanguan et al., 2006a). Hence, the lipase activity of the starter cultures; particularly at low pH should be further investigated. There have been many studies that have tried to reduce the fat content in fermented sausages (Liaros et al., 2009; Sriphochanart and Skolpap, 2011) as determined by the weight loss. However, the weight loss of GABA Nham was not significantly different from the other samples (Table 4.11).

The acidity resulted in protein coagulation and contributed to the firmness and color of the fermented meat product (Visessanguan et al., 2004); therefore a higher acidity in GABA Nham may have an influence on the texture and color of Nham products when compared with other Nham products (Table 4.11). Wiriyacharee et al., (1995) reported that *K. varians*, formerly known as *M. varians* was important in converting nitrate to nitroso-haemoglobin (NOMb), thus imparting a pink color to Nham. This indicated that the starter cultures and MSG decreased the NOMb by supporting LAB that grew faster than *K. varians*. In addition, the decrease of NOMb may be caused by lipid oxidation, and the accumulated hydrogen peroxide formed by the lactobacilli; and afterwards has been attributed to the paleness of the Nham during prolonged fermentation (Visessanguan et al., 2006a).

With regard to the texture and some color in GABA Nham there were some differences found when compared to both controls (Table 4.11). Nonetheless, the most desirable properties of good quality Nham such as the texture of the sample are tenderness with the ingredients used being well mixed, a good sourness taste and a natural pink color that were all achieved in GABA Nham. The GABA Nham obtained the highest scores on the sensory test for all parameters tested (Table 4.12). This

means that both starter cultures used at a level of each of 6 log CFU/g produced a high GABA content in Nham that met desirable sensory and physicochemical criteria with no effect on the texture, flavor and sourness including the color; and this is a good sign for producing a novel Nham product. In contrast, *L. curvatus* at the same level of cell density used as for our starter cultures caused an unusual smell (off-flavor) in Nham (Visessanguan et al., 2006b). However, in order to produce GABA Nham on a commercial scale or transfer its production to industry, safety and health issues that could arise such as the presence of BA and also an ability to reduce cholesterol were further investigated in the next experiment.

3. Effects of starter cultures on levels of Bas and cholesterol in Nham

3.1. The BAs content in the raw material and Nham samples

Table 4.13 shows the level of the 7 BAs; β-phenylethylamine, tyramine, histamine, cadaverine, putrescine, spermidine and spermine in ground pork sample were 4.48, 5.06, 32.92, 2.45, 1.85, 1.12 and 2.23 mg/kg fresh matter, respectively. Among the BAs examined in ground pork, only β -phenylethylamine, cadaverine and spermine were significantly higher than in all finished Nham products (96 h fermentation) ($P < 0.05$) while there was no significant difference for spermidine at 96 h with the exception of the control A and GABA Nham (T_{SM}). However, the amounts of β-phenylethylamine, cadaverine and spermine significantly decreased in all Nham sets along with the longer fermentation time. In contrast, the rest of the BAs in all the finished Nham products were significantly higher than those found in the initial ground pork ($P < 0.05$). Histamine, tyramine and putrescine were major BAs found in all finished Nham products. Comparisons between both controls (spontaneous fermentation) and inoculated sets, showed a remarkable decrease of all the BAs content in the finished Nham products in all the inoculated sets ($P < 0.05$) with the exception of cadaverine. For example, the histamine content in both controls was 156 and 166 mg/kg fresh matter, but both inoculated sets contained only 45

mg/kg fresh matter. The mixed starter culture reduced β-phenylethylamine by 100%, histamine 72% and tyramine 36% (see details in Table 4.13).

The amount of each of the 3 major BAs (ß-phenylethylamine, cadaverine and spermine) in all fermented Nham sets was less than in the ground pork (Table 4.13). This may be either because the indigenous microbes or a mixed starter had the ability to reduce these amines. In addition, Table 4.13 also shows that a mixed starter was able to reduce almost all the BAs studied such as β-phenylethylamine by 100% and histamine by 72% in both inoculated Nham products compared to both control Nham products. This implies that a mixed starter was able to outgrow the autochthonous microbes that produced the amines and the end BAs content was completely governed by the microbes present and was not related to the MSG content in the Nham samples. Only three BAs (tyramine, histidine and putrescine) were increased during the fermentation. This is a similar result to that of Limsuwan et al., (2007) who found that putrescine, cadaverine and tyramine (histamine was not studied) were the main amines formed during Nham fermentation with a total amount of 383.89 mg/kg. This is a reflection of the effect of the hygienic status of the raw pork meat used. If the meat had been stored at 30ºC for 6 h or at 4ºC for 2 days the meat could be classified at low quality and had high levels of cadaverine and tyramine; leading to the production of high levels of cadaverine, putrescine, tyramine and histamine in Nham products (Tosukhowong et al., 2011). This means that raw pork meat we used may have a higher quality based on the amounts of the 7 BAs (Table 4.13).

As the ability to reduce the BAs was dependent on the genera and strains, Zhang et al., (2013) reported that *L. plantarum* ZY-40 reduced the amount of putrescine and cadaverine during the fermentation of silver carp sausage by more than 70%, but did not reduce tyramine accumulation. On the other hand, our starter cultures reduced all the BAs studied and tyramine by 36% but with one exception for cadaverine compared with both the control fermentation. However, cadaverine in both the inoculated sets (roughly 2 mg/kg fresh matter) was lower than for the initial ground pork (2.45 mg/kg fresh matter). Shalaby (1996) recommended that the BA content in mg/kg in the processed sausage, according to “Good Manufacturing Practice”, should be in the range of 50-100 for histamine; putrescine, 30; cadaverine,

25; β -phenylethylamine, 30 and tyramine, 100-800. Hence, our inoculated Nham products met the requirement for BAs in sausage products.

3.2. Cholesterol content in Nham samples

Table 4.14 shows the amount of cholesterol in Nham samples (control: T_{NN} and GABA Nham: T_{SM}) at the beginning and at the end of the fermentation process (96 h). The amount of cholesterol at either the start or end of the fermentations in GABA Nham that used the mixed starter (*L. namurensis* NH2 and *P. pentosaceus* HN8) was significantly lower ($P < 0.05$) than that found in the control (no added starter T_{NN}). The finished products either in the control or GABA Nham sets had a lower cholesterol content at the end compared to the beginning of the fermentation ($P < 0.05$). The amount of cholesterol in the finished Nham products in the GABA Nham (T_{SM}, 96 mg/100 g fresh matter) was significantly lower than for the control set (T_{NM}, 149 mg100 g fresh matter) ($P < 0.05$). However, cholesterol content in the control Nham set was significantly higher than that in the commercial Nham brands (Table 4.14). No significant differences for the amount of cholesterol in both commercial Nham brands (125 and 114 mg/100 g fresh matter).

The mixed starter significantly reduced the cholesterol content in GABA Nham as its content in GABA Nham at the starting fermentation (t = 0) suddenly reduced (17.46%) and 35.42% at 96 h fermentation in the presence of the starter cultures (Table 4.14). This indicated that cholesterol might become quickly bound to the inoculant cells for a limited time and also other mechanisms should be involved during fermentation. This is supported by the suggestion of Hwang et al., (2011) in that the cholesterol might be removed by either binding to the bacterial cellular surface or by being absorbed into the cells. Other mechanisms to reduce cholesterol in addition to the cell attachment might be possible. In our previous study, both starter cultures could reduce cholesterol (31- 37%) in an *in vitro* test (Ratanaburee et al., 2013a); however, the mechanisms involved in the removal of cholesterol have not, so

far, been investigated. Several mechanisms have been hypothesized, which include enzymatic deconjugation of bile acids by a bile-salt hydrolase, assimilation of cholesterol, co-precipitation of cholesterol with deconjugated bile, cholesterol binding to cell walls, incorporation of cholesterol into the cellular membranes during growth, conversion of cholesterol into coprostanol and production of short-chain fatty acids upon fermentation (Gilliland et al., 1985; Noh et al., 1997; Ahn et al., 2003; Lye et al., 2010; Ooi and Liong, 2010). Hence, to understand the mechanisms used by our starter cultures to remove cholesterol in Nham, will be further investigated.

Table 4.13 Changes of biogenic amine concentrations in ground pork, Nham samples at the beginning and at the end of the fermentations.

Sample	Fermentation time (h)	Biogenic amine (mg/kg fresh matter)						
		β-Phenylethylamine	Tyramine	Histamine	Cadaverine	Putrescine	Spermidine	Spermine
Meat	ground pork	4.48 ± 0.02^{a}	5.06 ± 0.22^{e}	32.92 ± 0.32^{i}	2.45 ± 0.07^{a}	1.85 ± 0.02^{h}	1.12 ± 0.01^{e}	2.23 ± 0.05^{a}
Control A (T_{NN})	0	3.44 ± 0.09^{b}	2.21 ± 0.00^{h}	130.31 ± 0.45^{d}	ND	9.63 ± 0.06^{e}	1.20 ± 0.01^{b}	1.30 ± 0.03^{b}
	96	1.75 ± 0.01^{e}	16.47 ± 0.28^{a}	165.92 ± 0.50^{a}	ND	13.97 ± 0.13^{a}	1.16 ± 0.00^{c}	0.96 ± 0.01^{g}
Control B (T_{NM})	0	2.84 ± 0.03^{d}	4.27 ± 0.06^{f}	148.83 ± 0.09^{c}	ND	9.51 ± 0.07^{g}	1.19 ± 0.01^{a}	1.17 ± 0.02^{c}
	96	1.55 ± 0.02^{f}	15.35 ± 0.32^{b}	156.19 ± 0.11^{b}	ND	12.99 ± 0.04^{b}	1.13 ± 0.02^{e}	1.06 ± 0.04^{d}
T_{SN}	0	3.36 ± 0.05^{c}	2.07 ± 0.03^{i}	75.80 ± 0.13^{f}	ND	9.51 ± 0.13^{g}	1.10 ± 0.04^{f}	1.05 ± 0.02^{e}
	96	ND	11.25 ± 0.09^{c}	44.92 ± 0.07^{h}	2.17 ± 0.05^{b}	10.98 ± 0.17^{c}	1.12 ± 0.01^{e}	0.84 ± 0.00^{h}
GABA (T_{SM})	0	2.83 ± 0.05^{d}	2.80 ± 0.02^{g}	84.82 ± 0.16^{e}	ND	9.61 ± 0.01^{f}	1.14 ± 0.03^{d}	1.03 ± 0.02^{f}
	96	ND	9.04 ± 0.22^{d}	44.96 ± 0.11^{g}	2.12 ± 0.02^{c}	10.86 ± 0.07^{d}	1.16 ± 0.00^{c}	0.96 ± 0.04^{g}
MDL		0.25	0.15	0.12	0.11	0.09	0.11	0.13
% Biogenic amines reduction[A]		100	36.2	72.1	12.2[B]	19.0	<1	10.9

ND: Not detectable; MDL: Minimal detectable limit. Different lowercase letters in the same column indicate significant differences at $P < 0.05$, n = 3;

[A]Based on average values of both controls and both inoculated sets from finished Nham products;

[B]Based on average values of ground pork and both finished Nham products of inoculated sets.

Table 4.14. Effects of the mixed starter culture and fermentation time on the cholesterol content in GABA Nham and a control set, and comparison their finished Nham products with popular commercial Nham brands (different starter cultures were used).

Nham samples	mg cholesterol/100g Nham	
	0 h	96 h
Control (T_{NN})	$157.96 \pm 2.16^{a, A}$	$148.90 \pm 4.87^{a, B, 1}$
GABA Nham (T_{SM})	$130.41 \pm 3.61^{b, A}$	$96.15 \pm 4.76^{b, B, 3}$
Commercial Nham A	124.74 ± 3.47^{2}	
Commercial Nham B	113.83 ± 4.68^{2}	

Mean values of three determinations and their standard deviations are presented. For GABA and control Nham samples, different lowercase and uppercase letters are used to indicate significant differences in the same column and the same row, respectively ($P < 0.05$). Different superscripts of Arabic numbers in the same column are used to indicate significant differences among finished Nham products, ($P < 0.05$).

Overall the results of this study have demonstrated that the use of a mixed starter culture to produce a GABA Nham, had other benefits including the reduction of cholesterol by 35% when compared with the control Nham (Table 4.14). An increasing percentage of the cholesterol might be removed by increasing the numbers of LAB in the starter cultures to more than the 10^6 cells/g used in this study or with other methods after the mechanisms of cholesterol removal become known. Indeed, the amount of cholesterol (96 mg/100g fresh matter) in GABA Nham could be ranked as a moderate level because the current percent daily upper value (DV) for cholesterol is 300 mg (Piironen et al., 2002); hence 100 g GABA Nham provides 32% DV of cholesterol. Normally, Nham products are low in fat because lean pork meat is used for their production; however, high fat may be derived from cooked pork rind due to the fact that the observed cholesterol content of low fat pork sausages was 43 mg/100

g and lower than the control sausage values of (94 mg/100 g) (Mallika and Prabhakar, 2011). Compared to commercial Nham brands, higher cholesterol content should have been found in our control Nham due to a higher cooked pork rind used in our recipe. However, a mixed starter culture significantly reduced cholesterol content in the GABA Nham and the amount of cholesterol was lower than that in the commercial Nham brands (Table 4.14).

4. Succession of LAB during Nham fermentations

4.1. Conventional method

The microbiological analysis to monitor the dynamic changes in the LAB populations responsible for Nham fermentations is presented in Table 4.15 and Figure 4.5 According to the numbers of LAB and their different morphologies, it was possible that all representative colonies (RC) isolated from each Nham sample were the main dominant LAB at that particular time. The numbers of different LAB isolates in both Nham controls (T_{NN} and T_{NM}) were less than in both starter culture Nham sets (T_{SN} and T_{SM}) (Table 4.15). Identification results for the 286 LAB isolates showed that *L. namurensis* was not detected in either control set throughout the fermentation, while *P. pentosaceus* and *P. acidilactici* were observed in all Nham sets (Figure 4.5). At the beginning of the Nham fermentation only pediococci were found except for the inoculated Nham sets that had *L. namurensis*; however, at 24 h an indigenous lactobacillus, *L. plantarum* was found only in both of the controls while both inoculated sets had only *L. namurensis*.

Both pediococci were found in all Nham sets throughout the fermentations and more variety of lactobacilli was found with the longer fermentation times. Unidentified lactobacilli were observed only in both Nham control sets at both the 72 and 96 h fermentation samples. Among the 286 LAB isolates overall there were only 9 species of identified and unidentified lactobacilli (Figure 4.5 and Table 4.15). Amongst the dominant species throughout the fermentations, the most frequently found were *P. pentosaceus* 35.31%, followed by *L. namurensis* 19.23% (only in the inoculated Nham sets), *P. acidilactici* 15.03%, *L. plantarum* 14.69% and *L. brevis*

9.09%. Based on the frequency found the following species; *P. acidilactici*, *L. sake*, *L. curvatus* and *L. rhamnosus* were not different among the Nham sets. However, *L. plantarum*, *L. brevis* and an unidentified lactobacillus were more frequently observed in both controls without added starter cultures. In contrast, the more frequently found species found in both inoculated sets were *P. pentosaceus* and *L. namurensis*; therefore they were both able to compete effectively with the indigenous LAB. As shown, the only species of LAB that were common to all Nham sets included *P. pentosaceus, P. acidilactici, L. plantarum* and *L. brevis*.

Table 4.15. Numbers of different lactic acid bacteria (LAB) isolated from Nham samples during fermentations for monitoring the distribution of LAB.

Nham samples	Representative colonies (RC)					
	0 h	24 h	48 h	72 h	96 h	Total
Control A (T_{NN})	6	10	12	15	15	58
Control B (T_{NM})	7	10	13	15	15	60
T_{SN}	11	12	19	20	18	80
GABA (T_{SM})	12	16	20	20	20	88
Total	36	48	64	70	68	286

Table 4.16. Summary of the lactic acid bacteria (LAB) species found in Nham samples during fermentations for 96 h on the basis of conventional methods.

LAB	Number of LAB isolates				
	Control A (T_{NN})	Control B (T_{NM})	T_{SN}	T_{SM}	Total
P. pentosaceus	21^{b}	23^{b}	28^{a}	29^{a}	101 (35.31%)
L. namurensis	$-^{b}$	$-^{b}$	27^{a}	28^{a}	55 (19.23%)
P. acidilactici	11^{a}	11^{a}	10^{a}	11^{a}	43 (15.03%)
L. plantarum	13^{a}	13^{a}	7^{b}	9^{b}	42 (14.69%)
L. brevis	8^{a}	8^{a}	5^{b}	5^{b}	26 (9.09%)
L. sake	$-^{a}$	1^{a}	1^{a}	2^{a}	4 (1.40%)
L. curvatus	2^{a}	$-^{a}$	1^{a}	2^{a}	5 (1.75%)
L. farciminis	$-^{a}$	1^{a}	1^{a}	1^{a}	3 (1.05%)
L. rhamnosus	$-^{a}$	$-^{a}$	$-^{a}$	1^{a}	1 (0.35%)
Unidentified	3^{a}	3^{a}	$-^{b}$	$-^{b}$	6 (2.10%)

Mean values with different lowercase letters in the same row indicate significant differences ($P < 0.05$).

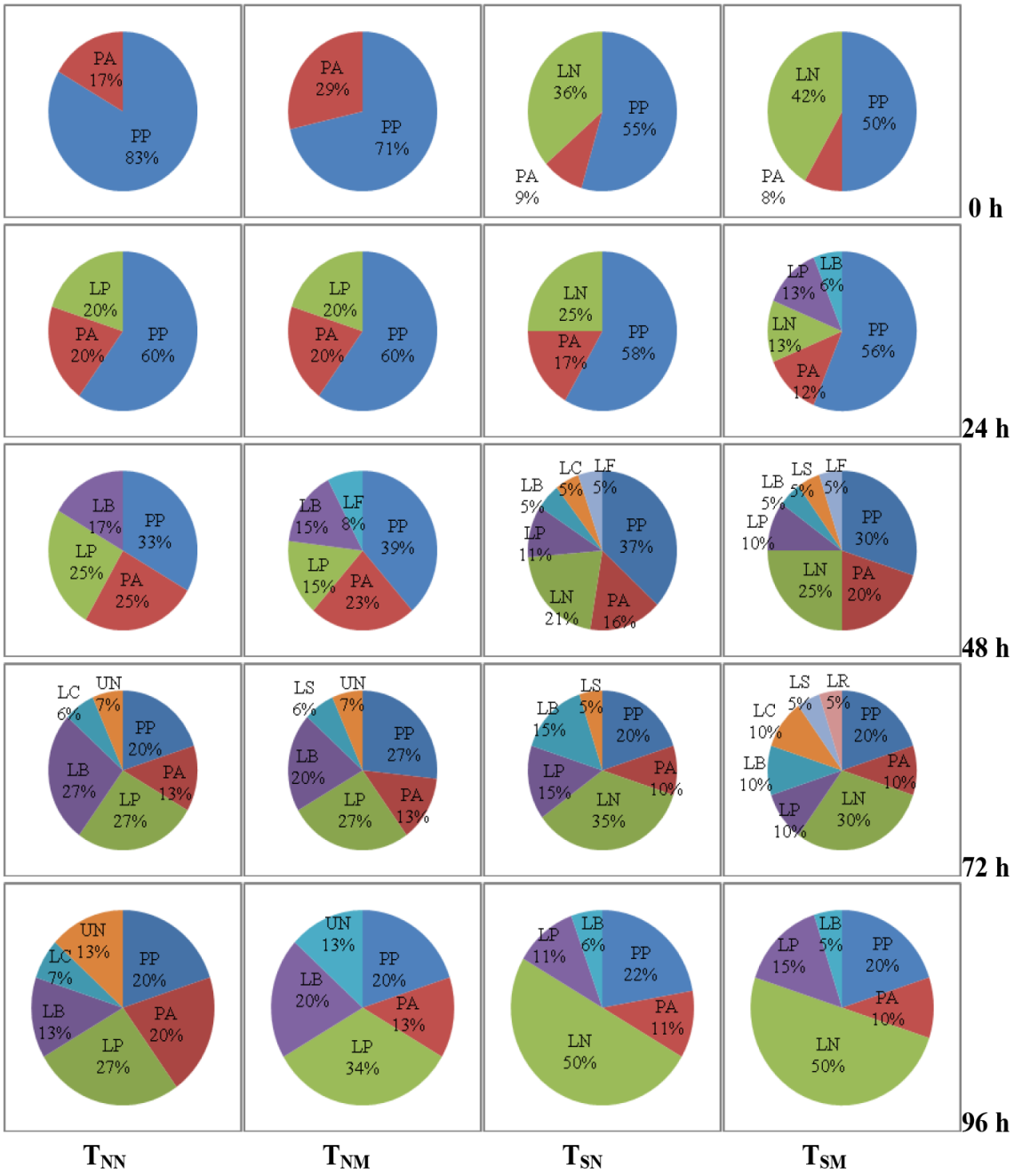


Figure 4.5. Distribution of lactic acid bacteria (LAB) in Nham samples (T_{NN}, No starter + No MSG; T_{NM}, No starter + 0.5% MSG; T_{SN}, starter + No MSG and T_{SM}, starter + 0.5% MSG) during 96 h fermentations. PP, *P. pentasaceus*; PA, *P. acidilactici*; LN, *L. namurensis*; LP, *L. plantarum* ; LB, *L. brevis*; LC, *L. curvatus*; LS, *L. sake*; LR, *L. rhamnosus* and UN, unidentified.

The distribution of LAB during Nham fermentations corresponded to their roles in reducing the amounts of BAs and cholesterol; this is because the amounts of BAs and cholesterol in both inoculated Nham products were significantly lower than that found in both controls without inoculation of a starter culture (Tables 4.15-4.16, Figure 4.5). The results in Figure 4.5 confirms that starter cultures played a role in reducing both the BAs and cholesterol contents in inoculated Nham products as in both controls *L. namurensis* NH2 was not detected throughout the fermentation time. However, to identify which of the starter cultures used might have a bigger role to reduce BAs and cholesterol; a single culture should be further investigated.

This study has confirmed that a mixed starter culture of *P. pentosaceus* HN8 and *L. namurensis* NH2 consistently produced 3,962 mg GABA/kg (data not shown) when compared with our previous study (4,051 mg GABA/kg) (section 3). This has also demonstrated that a mixed starter was the continuing dominant species during Nham fermentation, and emphasized that they were the best candidates for survival throughout the Nham fermentation process (Figure 4.5) as well as their ability to produce GABA and reduce the BAs and cholesterol. This is in agreement with the higher frequency of *L. plantarum*, *L. brevis* and an unidentified lactobacillus in the controls than that in the inoculated sets ($P < 0.05$) (Table 4.16) and this might reflect the role of *L. namurensis* or a mixed culture to have some control on the population of other indigenous lactobacilli. However, *L. plantarum* BCC9546 used as a commercial Nham starter culture significantly reduced 4 BAs (cadaverine, putrescine, histamine and tyramine) in Nham (Tosukhowong et al., 2011). This can be explained that in general the amino acid-decarboxylase activity depends on the strain not being species specific (Bover-Cid et al., 2001).

To explain why LAB isolates or RC in inoculated sets were higher that in the control sets (Table 4.15); this could be because no *L. namurensis* was found in the control groups (Figure 4.5 and Table 4.16). *P. pentosaceus* was the most dominant LAB (35.3%) found in all Nham products in this study (Figure 4.5 and Table 4.16). This is in agreement with Benito et al., (2007) who reported that in fermented meat dry sausages found that *P. pentosaceus* represented 31.2*%, L. lactis,* 26.9%, *P. acidilactici,* 18.6% and *L. brevis*.17%. There was a difference from our study for

lactobacilli excluding *L. namurensis*, in that *L. plantarum* was the most dominant isolate (14.7%) followed by *L. brevis* (9.1%) as shown in Table 4.16; this may be caused by different types of sausages because Nham is a fresh or wet sausage. This can be explained that different types of fermented meat products produced different dominant LAB as Tran et al., (2011) found a high prevalence of *L. plantarum* at 67.6% and the second predominant species was *P. pentosaceus* at 21.6% of LAB isolated from nem chua, a traditional uncooked Vietnamese fermented meat product.

4.2. DGGE community fingerprinting of Nham fermentations

Total DNA was extracted directly in order to obtain DGGE fingerprinting of the total LAB community without cultivation; and PCR-DGGE was performed by amplification of V3 region of 16S rRNA gene. The amplified fragments were in a range of less than 341 bp to 518 bp in size (Figure 4.6). These isolates can be identified as the individual strains due to they generated specific individual patterns (Figure 4.6 and Table 4.17). LAB associated with Nham samples (inoculated Nham and uninoculated Nham) during 96 h fermentation were identified as *L. plantarum* (band 3, 9, 12,17), *L. curvatus* (band 4,16, 26), *L. brevis* (band 5,18, 22, 27), *L. rhamnosus* (band 6, 21), *L. farciminis* (band 8, 20), *L. namurensis* (band 2, 7,11,15, 25), *Lactococcus lacti*s (band 13), *Pediococcus acidilactici* (band 14, 23), *P. pentosaceus* (band 1, 24) and *Weissella viridescens* (band 10,19). The results obtained from this study are in agreement with previous studies, which underline how lactobacilli and pediococci association largely dominates fermented sausages (Cocolin et al., 2001, 2004; Ercolini et al., 2006; Benito et al., 2007). Nguyen et al., (2013) reported that LAB associated with Nemchua were identified as *L. pentosus, L. plantarum, L. brevis, L. paracasei, L. farciminis, L. namurensis, Lactococcus lactis, P. acidilactici, P. pentosaceus, W. cibaria* and *W. paramesenteroides.* Among LAB, *L. sake, L. curvatus*, and *L. plantarum* are the species most frequently isolated in acid-fermented meat products (Aymerich et al., 2003; Rantsiou et al., 2005; Talon and Leroy, 2011) and this is in accordance with our study in Nham (Tables 4.16 and 4.17). It is well recognized that *W. viridescens* causes a negative effect in cooked meat

products by producing slime and greening of meat products; and then this leads to sensory defects (Duskova et al., 2013). In contrast, *L. lactis* has been reported for its potential to be used as a starter culture in fermented meat products (Noonpakdee et al., 2003).

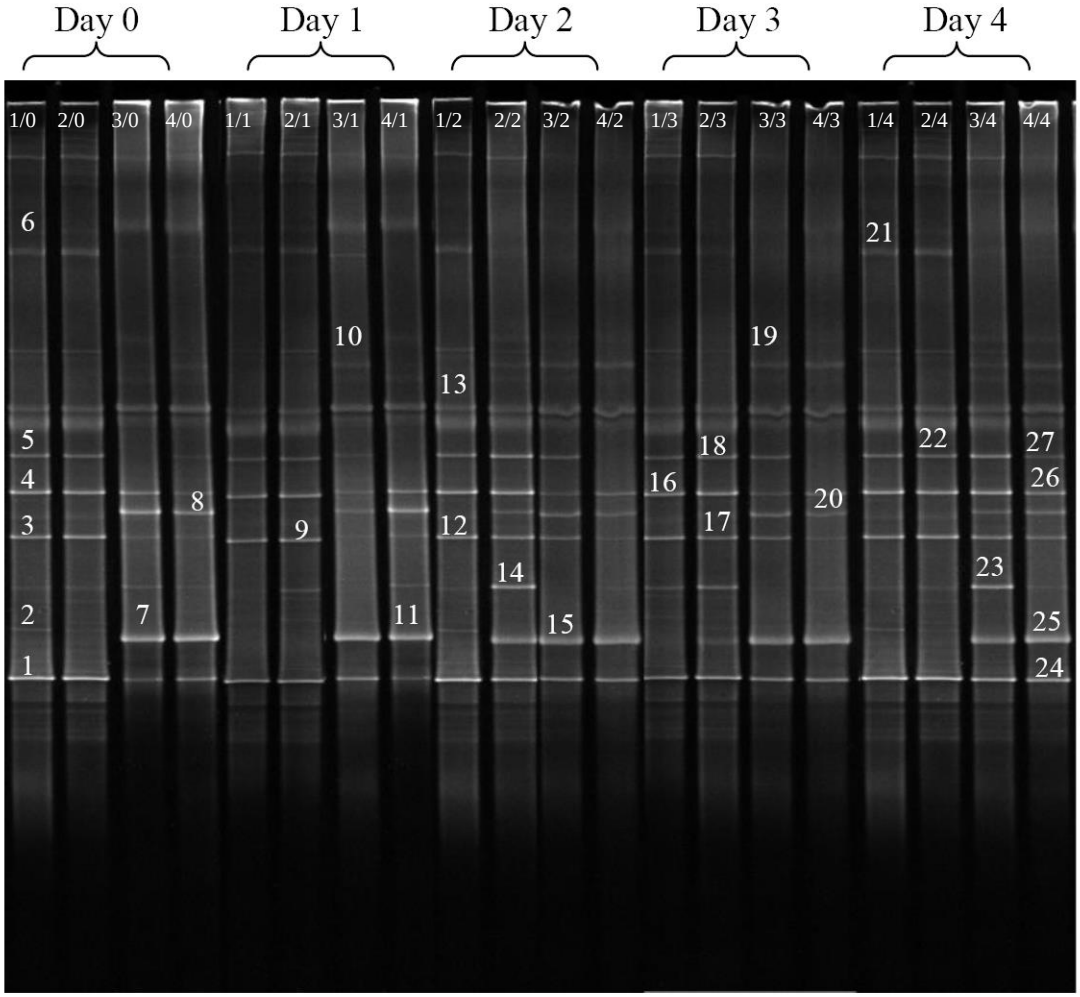


Figure. 4.6 DGGE profiles of LAB succession during 4 days in Nham fermentations between inoculated Nham (3 and 4) and uninoculated Nham (Control, 1 and 2). Lane designations indicate the fermentation type and the day of samplings. Lane 1/0-4/0, day 0; Lane 1/1-4/1, day 1; Lane 1/2-4/2, day 2; Lane 1/3-4/3, day 3; and Lane 1/4-4/4, day 4.

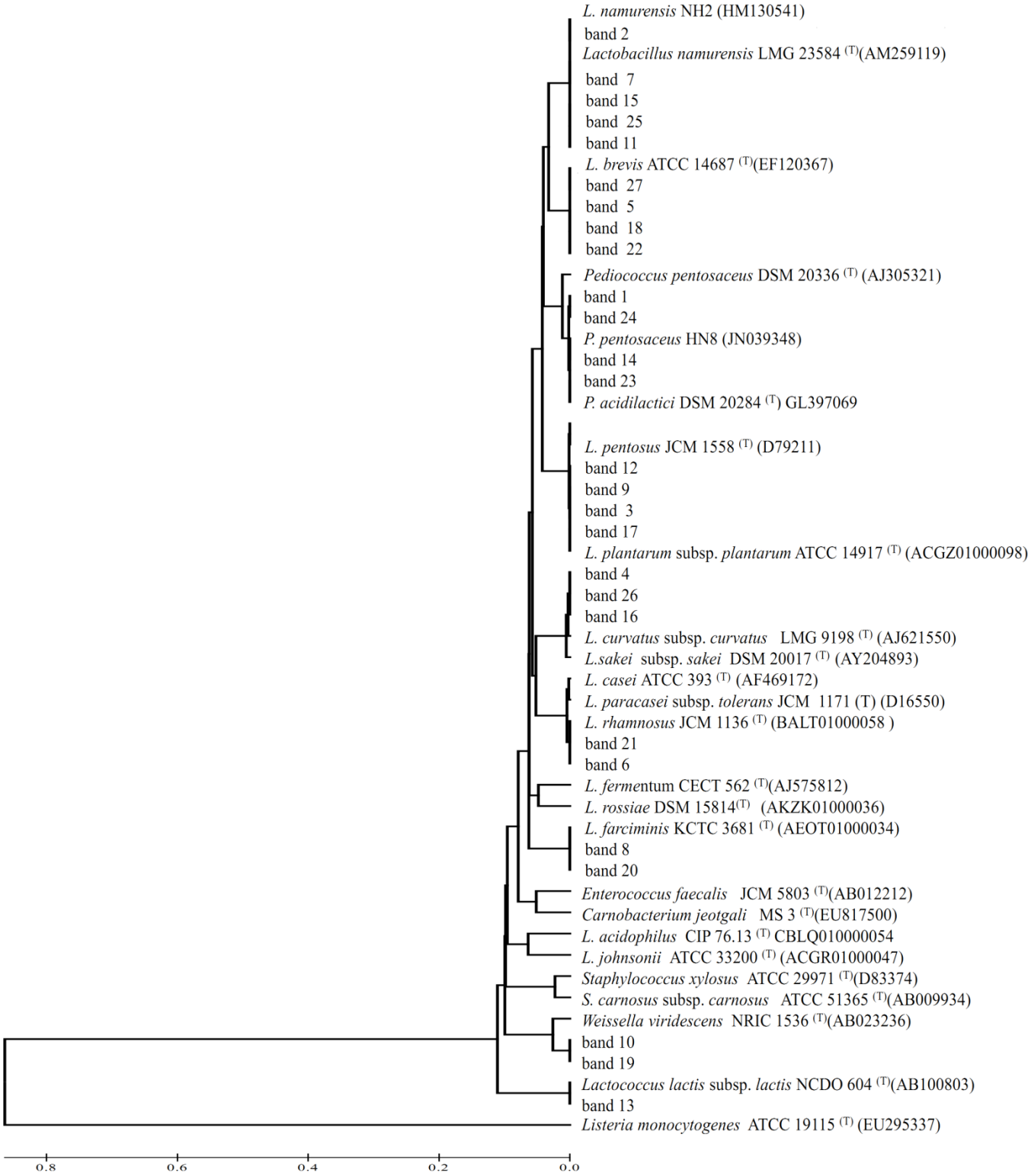


Figure 4.7. Dendrogram of LAB found in Nham fermentations for 4 days based on V3 sequences analysis. The phylogenetic tree was constructed with the MEGA 4 unweighted pair-group method using arithmetic averages.

Results of LAB community in Nham fermentations for 96 h using DGGE fingerprinting were compared with culture dependent of pure cultures. A similar result was found with a little difference. Biodiversity data of the LAB microbiota associated with the production of Nham obtained through the conventional cultivation-dependent approach did not fully correspond with the molecular inventory of the Nham samples through DGGE fingerprinting. For some isolated species, i.e. *L. namurensis*, and *W. viridescens*, were not found in the conventional approach of the uninoculated Nham samples or controls (Table 4.16); but *L. namulensis* was found in uninoculated Nham samples at days 2 and 3 by using PCR-DGGE (Table 4.17). Besides, *W. viridescens* and *L. lactis* were observed in all Nham fermentations throughout 96 h fermentation. However, the intensity of band for *L. lactis* was dense than that *W. viridescens*. This might indicate that a number of *L. lactis* was higher than that *W. viridescens.* According to band intensities, this may be a reason that both LAB genera were not detected by culture-dependent due to their less number when compared with starter cultures used and other detected LAB (Tables 4.16 and 4.17). On the other hand, it could be possible that unidentified isolates of pure cultures (Table 4.16) were both genera. Nevertheless, *L. sake* was only found with the use of culture-dependent and this organism has been reported as a common LAB found in fermented meat products as previously mentioned. Hence, it is possible that different species might be represented by bands that stop migrating at the same position in the gel leading to an underestimation of the LAB diversity. For example, *L. pentosus* and *L. plantarum*, two species which were predominantly present could not be discriminated in the DGGE fingerprints as they were represented by the same single band in DGGE profiles (Rantsiou et al., 2004). Conversely, the use of the 16S rRNA gene for DGGE analysis could introduce some biases as it is often present in multiple copies, i.e. the 16S rRNA gene operon heterogeneity, which results in multiple DGGE bands what could lead to an overestimation of the actual microbial diversity (Muyzer and Smalla, 1998).

Overall results proved the advantage of the comparative study by both culture-dependent and culture-independent techniques as both approaches provided sufficient data for evaluating LAB communities during Nham fermentations. According to culture-dependent and culture-independent methods showed succession

of LAB throughout fermentation for 96 h as a mixed culture *(P. pentosaceus* and *L. namurensis)* were found in both inoculated Nham samples while *L. namurensis* was rarely found in both controls (Figure 4.6). As their accession numbers were the same as our starter cultures (Figures 4.2 and 4.7); this means that both starter cultures could survive to be dominant organisms during Nham fermentations. In addition, based on DGGE profiles succession of native LAB was similar among Nham fermentations for 96 h; and this is the reason that why inoculated Nham, particularly GABA Nham showed a better quality than other Nham products by consisting of high amount of GABA, but less in cholesterol and BAs as previously described.

Table 4.17. DNA sequencing results of LAB communities found in Nham samples based on the cut bands from the DGGE gels (Figure 4.6).

Band (s) [a]	Closest relative	% Identity [b]	Accession No. [c]
1, 24	*Pediococcus pentosaceus*	100	JN039348
2, 7, 11, 15, 25	*Lactobacillus namurensis*	100	HM130541
3, 9, 12, 17	*L. plantarum*	100	KM350173
4, 16, 26	*L. curvatus*	100	KJ806306
5, 18, 22, 27	*L. brevis*	100	KJ584843
6, 21	*L. rhamnosus*	100	KM350174
8, 20	*L. farciminis*	100	NR 114398
10, 19	*Weissella viridescens*	100	HE575179
13	*Lactococcus lactis*	100	KF245554
14, 23	*P. acidilactici*	100	KM062019

[a] Bands are numbered as indicated on the DGGE gels shown in Figures 4.6 and 4.7.

[b] Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank database.

[c] Accession number of sequence of the closest relative found by BLAST search

CHAPTER 5

CONCLUSIONS

As this thesis focused to add value into a fermented ground pork sausage (Nham) for producing Nham that enrich with GABA by using our own starter cultures, and also to know the role of starter cultures used; the finding of the research works are concluded from each aim as follows.

1. Until now, starter cultures have been used to improve the quality of Nham by obtaining consistency of the product and controlling pathogens; however, in this study we obtained two GABA-producing LAB strains (*P. pentosaceus* HN8 and *L. namurensis* NH2) that seemed to have all the right probiotic properties. Hence, they will be used as starter cultures for producing a novel fermented meat product.

2. The results have shown that the starter cultures consisted of *P. pentosaceus* HN8 and *L. namurensis* NH2 (ratio 1:1), each added at 10^6 CFU/g with the addition of 0.5% MSG significantly enhanced the GABA content in Nham. A unique Nham product with the best sensory evaluation was developed with a high amount of GABA but low in fat, carbohydrate and energy; therefore it can be considered to be a novel Nham product.

3. The amounts of cholesterol and BAs in the fermented meat products was dependent on the raw material used and the microbes involved; however, these compounds can be reduced by using a proper starter culture. This study has proven that a GABA Nham with lower amounts of BAs and cholesterol to be in safe levels could be achieved by using the starter cultures as mentioned above. Hence, a safe healthy fermented pork sausage has been successfully developed.

4. To ensure the role of starter cultures in Nham fermentations, LAB succession was investigated by both culture-dependent and culture-independent methods to obtain data for interpreting the role of starter cultures. The results showed that both promising strains (HN8 and NH2) were dominant throughout Nham

fermentation for producing a novel Nham product. Hence, quality improvement of fermented meat products like Nham could be possible with the use of a mixed culture.

Future research needs

Regarding to the findings of this thesis, there are many questions that remain with no answers and some specific suggestions that arising from the current studies are the following provided.

1. To identify which of the starter cultures used (HN8 or NH2) might play a bigger role to reduce cholesterol and BAs in Nham; a single culture should be further investigated.

2. Removal mechanisms of each starter culture should be studied *in vitro* as it would help to explain why inoculated Nham had lower amount of cholesterol than that unionoculated Nham.

3. Can a novel GABA Nham reduce cholesterol content in blood pressure? This should be studied *in vivo* to ensure the benefit of consuming the GABA Nham product.

4. Molecular work other than DGGE should be used to study bacterial community in Nham to obtain more native species in Nham fermentation.

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APPENDIX

A. Medium

de Man Rogosa and Sharpe agar (MRS agar) (Merck)

Peptone from casein	10.0	g/L
Meat extract	8.0	g/L
Yeast extract	4.0	g/L
D(+)Glucose	20.0	g/L
Tween 80	1.0	g/L
di-Ammonium hydrogen citrate	2.0	g/L
di-Potassium hydrogen phosphate	2.0	g/L
Sodium acetate	5.0	g/L
Magnesium sulfate	0.2	g/L
Manganeses sulfate	0.04	g/L
Agar	14.0	g/L
Distilled water	1	L

The MRS agar was prepared by suspending 68.2 g in 1 L of distilled water and autoclaving for 15 min at 121°C. The medium was allowed to cool to 45-50°C, and used before the surface of the plates had dried, following the manufacturer's instructions.

de Man Rogosa and Sharpe broth (Merck)

Peptone from casein	10.0	g/L
Meat extract	8.0	g/L
Yeast extract	4.0	g/L
D(+)Glucose	20.0	g/L
Tween 80	1.0	g/L
di-Ammonium hydrogen citrate	2.0	g/L
di-Potassium hydrogen phosphate	2.0	g/L
Sodium acetate	5.0	g/L
Magnesium sulfate	0.2	g/L
Manganeses sulfate	0.04	g/L
Distilled water	1	L

The MRS broth was prepared by suspending 52.2 g in 1 L of distilled water and autoclaving for 15 min at 121°C.

B. Chemical Analysis

1. The amount of GABA in Nham

There are two steps for determination amount of GABA in Nham samples.

- Nham samples were extracted to release GABA by 4% acetic acid (Nham and 4% acetic acid ratio 1: 4).

- Amount of GABA in Nham was determined by High-Performance Liquid Chromatography (HPLC) as follows.

- The samples were collected and centrifuged at 1,500 xg for 15 min. The supernatant was derivatized with PITC (phenylisothiocyanate) and then filtered through a 0.45μm filter, and analyzed by HPLC (Hewlett Packard 1100 series).

- GABA analysis was performed using a HPLC equipped with a Waters symmetry C_{18} column (4.6 x 250 mm, 5 μm), a HP 1100 series binary pump, a HP 1100 series autosampler, column oven (46^{o}C), and a HP 1100 series UV detector (254 nm).

- The elution solvent system was comprised of (A) 1.4 mM NaHAc, 0.1% TEA, and 6% CH_3CN (pH 6.1), and (B) 60% CH_3CN.

- The column was eluted for 50 min with a linear gradient of 0-100% at 1.0 mL/min with (B).

Standard curve and HPLC chromatogram for GABA peak are presented in Figures 1 and 2.

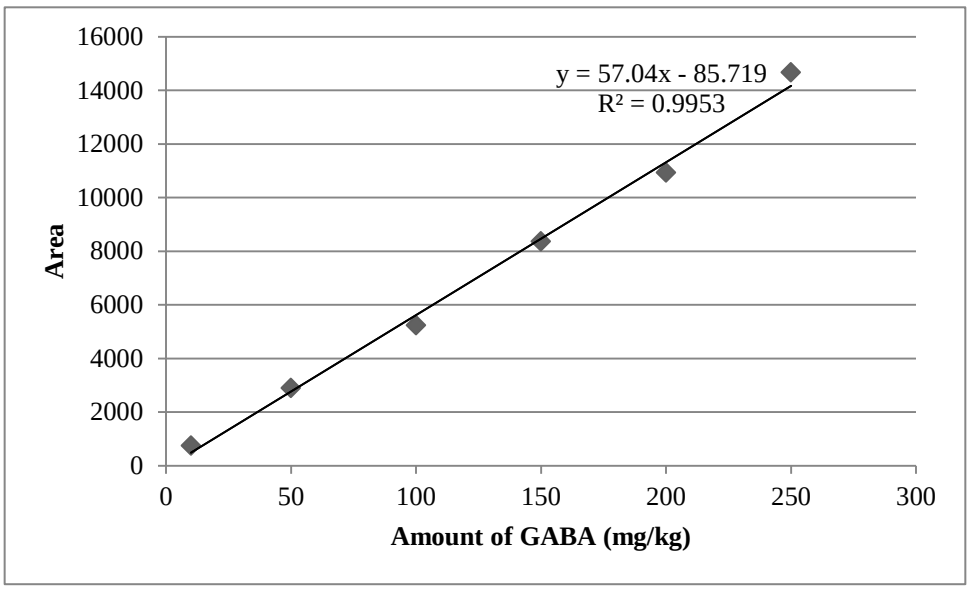

Figure 1. A standard curve illustrating the stability of the phenylisothiocyanate derivative/amino acid complex formed. The slope was determined as having a linear gradient of 0.9953

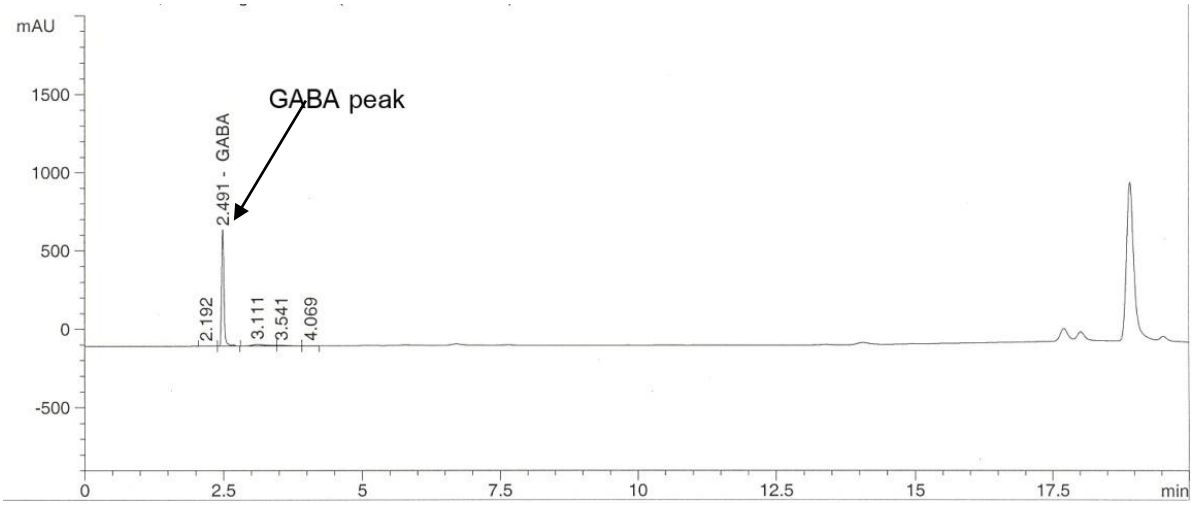

Figure 2. HPLC profile of γ- aminobutyric acid (GABA)

2. Analysis of Cholesterol in Nham samples

The method of Moses et al., (1975) was adopted with some modification as follows.

- Nham samples were weighed (1 g for each sample) and placed into a 125 mL boiling flask, followed by 250 mg of pyrogallol and 50 mL of 95% ethanol, and then 25 mL of 60% KOH.

- The mixture was stirred, boiled, and refluxed for 30 min, as compared to 75-80°C in the original procedure followed by cooling at 25°C. A 1 min mixing was required before the solution was transferred to a 250 mL separating funnel, 50 mL of hexane and 25 mL of distilled water were added into each separating funnel. At least twice washes of hexane extract were used to eliminate aqueous components.

- After incubation at room temperature for 5 min to allow phase separation, the final hexane layer was transferred into a clean flask containing anhydrous sodium sulfate; so that all the moisture associated with hexane was removed. The hexane layer was collected and evaporated at 40°C.

- The stock reagent was prepared by dissolving 0.1 g of $FeCl_3.6H_2O$ in 5 mL of deionized water and 95 mL of glacial acetic acid using a 100 mL volumetric flask. Prior to use, 86 mL of concentrated H_2SO_4 was added. The stock solution (2 mg/mL) was prepared by dissolving 200 mg of standard cholesterol (chromatography grade; Sigma Chemical, USA) in 1-propanol using a 100 mL volumetric flask.

- The stock solution was used to prepare working solutions containing 0.05, 0.10, 0.15 0.20 and 0.25 mg cholesterol/mL. A 3 mL of the $FeCl_3$ coloring reagent was added into a total volume of each working solution (1 mL), and the developed color was measured at 540 nm using a spectrophotometer.

A standard curve was built by using the standard concentration of cholesterol for the abscissa and the absorbance of each working solution for the ordinate (Figure 3).

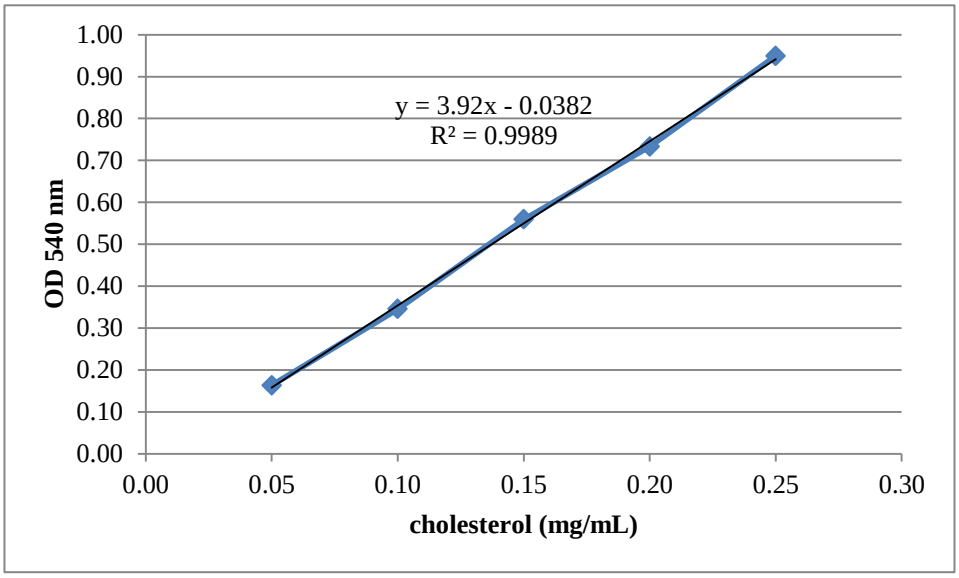

Figure 3. A standard curve of cholesterol. The slope was determined as having a linear gradient of 0.9989.

3. Analysis of the BAs of raw material and Nham samples

BAs were determined by HPLC according to methods described by Tosukhowong et al., (2011) and Önal et al., (2013). In this study, 7 BAs; tyramine, putrescine, cadaverine, histamine, β-phenylethylamine, spermidine and spermine were determined as follows.

- All BA standards were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. and separately prepared in deionized water at a concentration of 1,000 mg/L.

- Each 5 g of Nham samples or ground pork were used to extract BAs by adding 15 mL of 0.4 M perchloric acid containing 125 µL of 1, 7-diaminoheptane (10 mg/mL) as an internal standard and the mixture was homogenized for 5 min.

- The homogenate was centrifuged at 6,000 x*g* at 4ºC for 5 min. The supernatant was collected and the residue was extracted twice with 10 mL of 0.4 M perchloric acid solution. All supernatants were combined and the final volume was adjusted to 25 mL with 0.4 M perchloric acid. The extract was filtered with a Whatman No.1 filter paper.

- Adding 60 µL sodium hydroxide (2 N, NaOH), 90 µL of saturated sodium bicarbonate and 600 µL of a dansyl chloride solution (10 mg/mL) into the extracted sample or standard solution (300 µL).

- After incubation at 40ºC for 45 min in the dark, 30 µL of 25% (v/v) ammonia solution was added to the reaction mixture for the removal of residual dansyl chloride. This reaction mixture was centrifuged for 5 min at 3,500 x*g*. The supernatant was filtered through a 0.45 µm syringe filter with a PVDF Membrane for HPLC analysis.

- A Zorbax Eclipse XDB C_{18}, (150 x 4.6 mm inner diameter, 5 µm) column was used with the mobile phase acetonitrile containing 0.1% (v/v) acetic acid (solvent A) and 0.1% (v/v) acetic acid (solvent B) at a flow rate of 1.5 mL/min. The column was equilibrated with 45% solvent A and 55% solvent B for 38 min before the next injection. The column was kept at 40°C in a heated column compartment. A 20 µL sample was injected, and the dansylated amines were detected at 254 nm with 550 nm as the reference.

Figure 4 shows the HPLC chromatogram of BAs. As there were 7 standard curves to cover all BAs tested; however, only standard curve of putrescine is presented as the example in Figure 5.

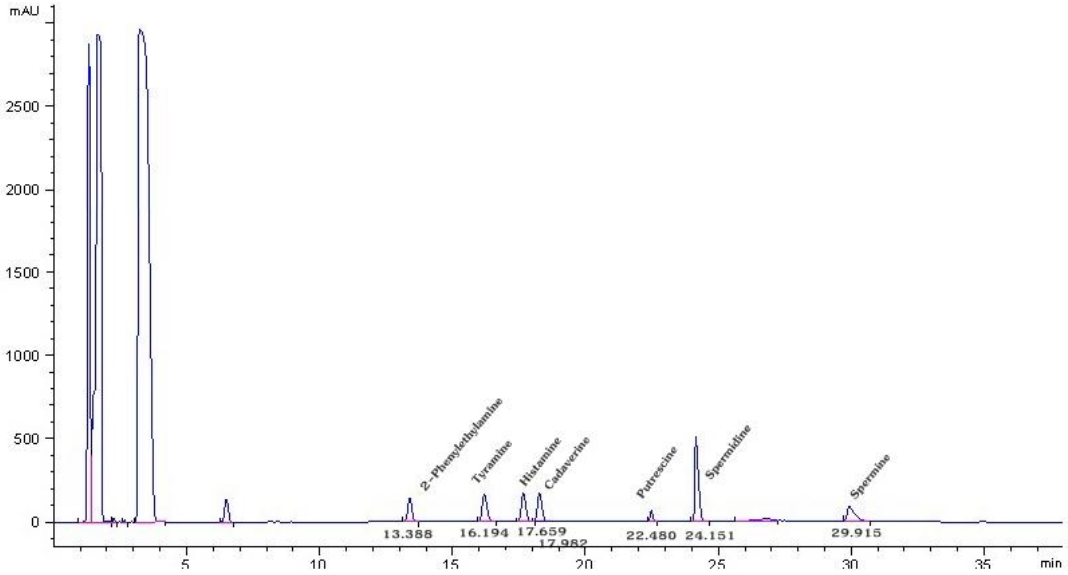

Figure 4. HPLC chromatographic profiles of the dansyl chloride-derivatives of a biogenic amine standard solution. Peak identities: β-phenylethylamine, tyramine, histamine, cadaverine, putrescine, spermidine and spermine.

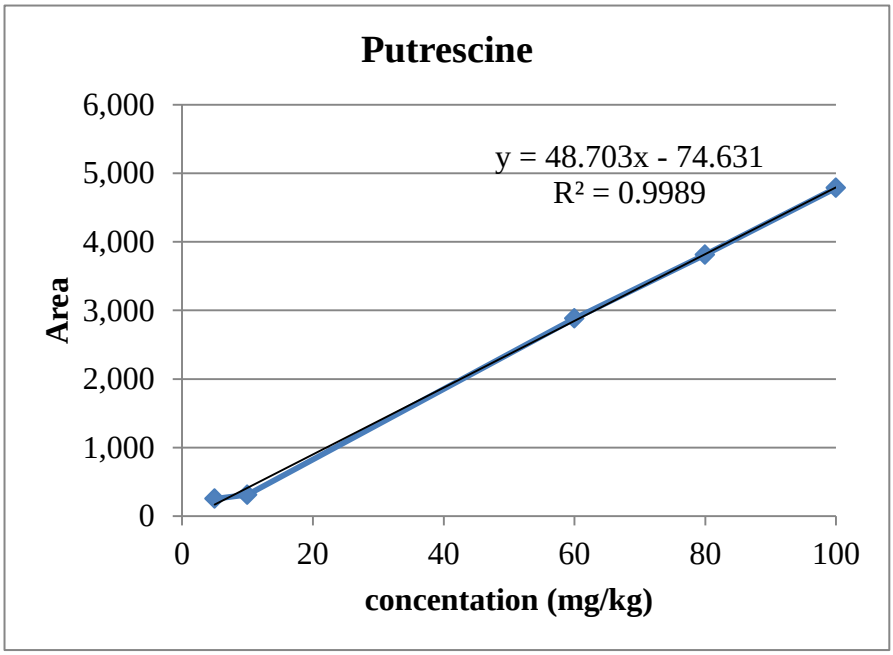

Figure 5. A standard curve of putrescine. The slope was determined as having a linear gradient of 0.9989

C. Report of microbial identification by partial 16S rDNA sequence analysis
Sample Name : NH2

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898 bp Identification

Homology Search with BLASTn program from NCBI database

Sequences producing significant alignments:		SCORE	E VALUE
HM130541	*Lactobacillus namurensis* strain Ln-15	1620	0.0
AB626072	*Lactobacillus namurensis* strain: NBRC 107158	1615	0.0
AM259119	*Lactobacillus namurensis* strain LMG 23584T	1615	0.0
AM259118	*Lactobacillus namurensis* strain LMG 23583T	1615	0.0
AB626069	*Lactobacillus spicheri* strain: NBRC 107155	1579	0.0

BLASTN 2.2.25+
Reference:
Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 3R7SE6DM012

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
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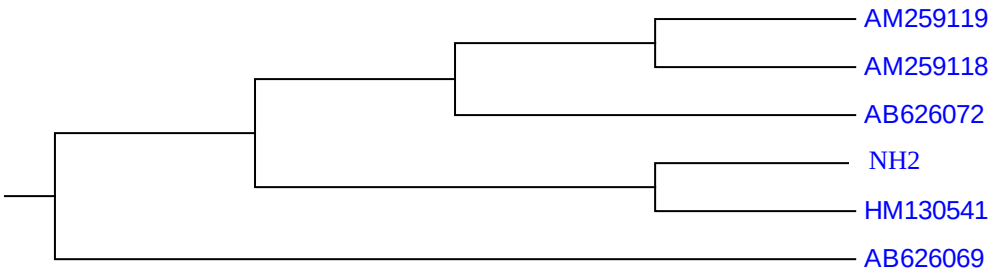
Alignment and Phylogenic tree by MEGA 4 : Unweighted pair-group method using arithmetic averages (UPGMA)



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Identities = 898/898 (100%), Gaps = 0/898 (0%)
Strand=Plus/Plus

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LOCUS HM130541 1476 bp DNA linear BCT 30-MAY-2010
DEFINITION *Lactobacillus namurensis* strain Ln-15 16S ribosomal RNA gene, partial sequence.
ACCESSION HM130541
VERSION HM130541.1 GI:296788341
KEYWORDS .
SOURCE *Lactobacillus namurensis*
ORGANISM *Lactobacillus namurensis*
Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.
REFERENCE 1 (bases 1 to 1476)
AUTHORS Sheng,H.-Y., Guo,Y.-P., Chang,Y. and Zhang,M.
TITLE The biodiversity of lactic acid bacteria from the traditional fermented vegetable
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1476)
AUTHORS Sheng,H.-Y., Guo,Y.-P., Chang,Y. and Zhang,M.
TITLE Direct Submission
JOURNAL Submitted (16-APR-2010) College of Life Science, Anhui Agriculture
University, Changjiang West Road, Hefei, Anhui 230036, China
FEATURES Location/Qualifiers
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/organism="Lactobacillus namurensis"
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ORIGIN

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    241 agcttgttgg tggggtaacg gctcaccaag gcgatgatgc gtagccgacc tgagagggta
    301 atcggccaca ttgggactga gacacggccc agactcctac gggaggcagc agtagggaat
    361 cttccacaat ggacgcaagt ctgatggagc aatgccgcgt gagtgaagaa gggtttcggc
    421 tcgtaaaact ctgttgttga agaagaacgg gtgtcagagt aactgttgac atcgtgacgg
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Sample Name : HN8

---

905 bp Identification

Homology Search with BLASTn program from NCBI database

Sequences producing significant alignments:		SCORE	E VALUE
JN039348	*Pediococcus pentosaceus* strain LAB2	1633	0.0
HQ711362	*Pediococcus acidilactici* strain L3-1	1633	0.0
HQ711356	*Pediococcus acidilactici* strain L6-1	1633	0.0
JF268323	*Pediococcus acidilactici* strain 0-4A	1633	0.0
GU904684	*Pediococcus acidilactici* strain L94	1633	0.0

BLASTN 2.2.25+

Reference:

Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 3R9ZS91Y01S

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 14,409,272 sequences; 37,064,856,589 total letters

Query= Length=905

>HN8 contig

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AGTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGA
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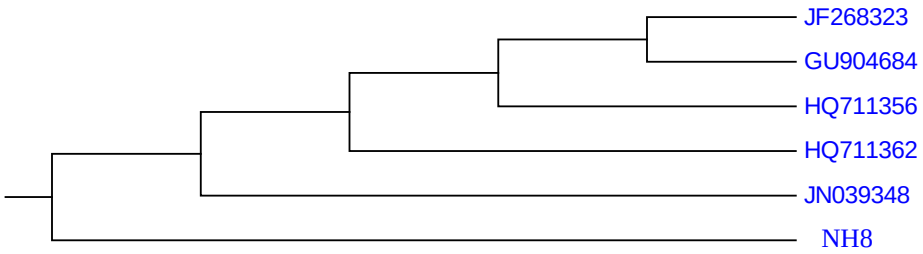
Alignment and Phylogenic tree by MEGA 4 :Unweighted pair-group method using arithmetic averages (UPGMA)



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Score = 1633 bits (1810), Expect = 0.0
Identities = 905/905 (100%), Gaps = 0/905 (0%)
Strand=Plus/Plus

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Sbjct  407   GGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCAC  466

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Query  241   GTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGA  300
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Query  301   ACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGG  360
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Sbjct  827   GAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGCCTGGGGAGTACG  886

Query  481   ACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG  540
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DEFINITION *Pediococcus pentosaceus* strain LAB2 16S ribosomal RNA gene, partial sequence.
ACCESSION JN039348
VERSION JN039348.1 GI:339736760
KEYWORDS.
SOURCE *Pediococcus pentosaceus*
ORGANISM *Pediococcus pentosaceus*
Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Pediococcus.
REFERENCE 1 (bases 1 to 1472)
AUTHORS Ramasamy,K. and Abdul Rahman,N.Z.
TITLE Isolation and characterization of lactic acid bacteria
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1472)
AUTHORS Ramasamy,K. and Abdul Rahman,N.Z.
TITLE Direct Submission
JOURNAL Submitted (01-JUN-2011) Pharmacy, Universiti Teknologi Mara, Puncak Alam, Selangor, Selangor 57000, Malaysia
COMMENT Sequences were screened for chimeras by the submitter using Bellerophon 3.0.
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/db_xref="taxon:1255"
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ORIGIN

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D. Sensory test form using Hedonic-5-scale (Thai Industrial Standard number 1219-2547)

Product: Nham

Name:……Date……Time……

Recommendation: Please examine the purpose samples from left to right and then score each factor according to your sensory.

Sensory properties				**Score**
Texture	**Flavor and sourness**	**Color**	**Overall acceptance**	
Tenderness and mixed well between meat and meat rind	Tasty aroma with a good sourness	Natural pink of nitrohaemochrome	Like extremely	5
Tenderness but not adequately mixed of meat and meat rind	Moderate fragrance and a good sourness	A little dark	Like	4
No cohesiveness but rigidity; however some crack found	Slight fragrance and sourness.	Pale color	Moderately acceptable	3
No cohesiveness and form including lack of homogenous	A little spoiled odor Very sour	Dark green	Dislike	2
Tender and no form	Smell and taste are not acceptable	Very dark green	Dislike extremely	1

Comments or suggestions:

……

……

…… ……..Thank you

VITAE

Name Miss Anussara Ratanaburee

Student ID 5310230040

Educational Attainment

Degree	Name of Institution	Year of Graduation
M.Sc. (Microbiology)	Prince of Songkla University	2010
B.Sc. (Microbiology)	Prince of Songkla University	2008

Scholarship Awards during Enrolment

The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Prince of Songkla University.

List of Publication and Proceedings

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W., Sukhoom, A., 2013. Selection of γ-aminobutyric acid-producing lactic acid bacteria and their potential as probiotics for use as starter cultures in Thai fermented sausages (Nham). International Journal of Food Science and Technology. 48: 1371-1382.

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W., Sukhoom, A. 2013. Enhancement of γ-aminobutyric acid (GABA) in Nham (Thai fermented pork sausage) using starter cultures of *Lactobacillus namurensis* NH2 and *Pediococcus pentosaceus* HN8. International Journal of Food Microbiology. 167: 170–176.

Duangporn Kantachote, Anussara Ratanaburee, Tawatchai Sumpradit and Ampaitip Sukhoom. Reduction of biogenic amines and cholesterol in Thai fermented pork sausage (Nham) by use of starter cultures and their behavior during the fermentation process. It will be submitted.

Ratanaburee, A., Kantachote, D. and Charernjiratrakul, W. 2011. Selection of lactic acid bacteria with ability to produce γ-aminobutyric acid (GABA) isolated from Thai fermented foods. The proceeding of the 21st National Graduate Research Conference. Rangsit University, Thailand. May 26, 2011

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ดวงพร คันธโชติ วิลาวัณย์ เจริญจิระตระกูล อำไพทิพย์สุขหอม และ อนุสรา รัตนบุรี. 2555. ผลิตภัณฑ์แหนมกาบา. การนำเสนอแบบโปสเตอร์โดยส่งบทคัดย่อ ในการประชุมสุดยอดมหาวิทยาลัยวิจัยแห่งชาติ ครั้งที่ 1 (NRU SUMMIT I). The 1st Thailand National Research University Summit. ระหว่างวันที่ 29-30 เมษายน พ.ศ. 2555 ณ ศูนย์ประชุมแห่งชาติสิริกิตติ์ กรุงเทพมหานคร

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1 สิงหาคม 2557 ณ บางกอกคอนเวนชั่นเซ็นเตอร์ ชั้น 22 โรงแรมเซ็นทาราแกรนด์ เซ็นทรัลเวิลด์ กรุงเทพฯ

Petty patent

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