Isolation and Selection of Cholesterol-lowering and γ-Aminobutyric Acid-producing Lactic Acid Bacteria for Producing Functional Fermented Dairy Products

Jirayu Jitpakdee

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Microbiology

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Major Program	Microbiology

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The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfilment of the requirements for the Master of Science Degree in Microbiology

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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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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ABSTRACT

Nowadays, functional foods are popular as high demand of consumers. The use of starter(s) from lactic acid bacteria (LAB) for functional food productions is one of alternative ways to produce functional foods. This research collected 60 samples of various fermented foods and raw milk in Thailand to isolate LAB. A total of 244 strains from 39 fermented foods were primarily screened using basal medium containing 100 µg/ml cholesterol and 0.1% yeast extract and found 139 strains survival. However, for secondary screening, only 10 strains survived in the same medium that yeast extract was removed. Five LAB strains (ENM104, ENM203, ENM204, NNM205 and NNM304) were passed tertiary screening as their proliferations. Of these, only ENM104 reduced cholesterol at 7.53 ± 1.78 % in basal medium supplemented with 100 µg/ml cholesterol-PEG600 at 72 h of fermentation. Screening of GABA producer, ten LAB isolates obtained from secondary screening found that only SPS109 strain in MRS fortified with 5 mg/ml monosodium glutamate (MSG) released 1,157.01 ± 4.76 µg/ml at 72 h of incubation. Selected LAB strains were identified using both biochemical test with 49 sugar fermentations of API 50 CHL test kit and molecular identification with 16S rRNA gene. Strains ENM104 and SPS109 showed the highest similarity with *Pediococcus pentosaceus* DSM20336^T at 99.65 % and *Lactobacillus plantarum* DSM20174^T at 99.93 %, respectively. Both LAB strains were investigated for probiotic properties. The results showed that none of strains hydrolysed starch, but only SPS109 hydrolysed tributyrin. However, both LAB strains hydrolysed casein. Based on bile salt hydrolysis, both strain hydrolysed glycocholic acid (GCA), taurocholic acid (TCA) and taurodeoxycholic acid (TDCA). Gastrointestinal tolerance was tested *in vitro* displayed that both strains survived in acidic condition mimicking from stomach with viable at 6.20 ± 0.12 and 7.39 ± 0.12 log CFU/ml (initial cells at 10.18 ± 0.02 and 10.46 ± 0.04

log CFU/ml) of ENM104 and SPS109, respectively. Furthermore, cells slightly increased in basic condition mimicking from intestine at 6.49 ± 0.00 and 7.63 ± 0.02 log CFU/ml, respectively. Cholesterol-lowering property testing in MRS containing 100 µg/ml of cholesterol-PEG600 found that ENM104 and SPS109 reduced 15.34 ± 1.12 and 10.05 ± 2.32 % at 72 h of incubation, respectively. After that, ENM104 in the same medium produced cholesterol oxidase and its specific activity was 8.73 ± 2.00 mU/mg protein after 72 h of incubation. ENM104 and SPS109 strains tolerated in 1 mM of H_2O_2 for 20 and 12 h, respectively. Antioxidant activity was also tested in MRS by ORAC in µmol TE/ml found 37.26 ± 0.77 at 48 h for ENM104 and 34.01 ± 3.30 at 72 h for SPS109. A similar result was observed for total phenolic content (TPC) at 72 h of incubation by both strains as 0.67 ± 0.00 and 0.68 ± 0.00 µmol GE/ml. For application in dairy products, single and co-culture (1:1) in milk/whey fermentation was investigated. In fermented milk, it found the lowest pH at 5.32 ± 0.01 in co-culture set at 72 h, and this set had population in log CFU/ml of ENM104 at 6.93 ± 0.01 and SPS109 at 8.27 ± 0.04. After 72 h incubation, cholesterol was reduced in fermented milk at 15.71 ± 0.64 % for ENM104 set and 10.98 ± 3.80 % for co-culture set. On the other hand, GABA was found at 4.57 ± 0.01 and 4.47 ± 0.04 µg/ml in SPS109 and co-culture sets, respectively. A co-culture set showed maximum at 57.63 ± 2.97 % inhibition against on angiotensin-converting enzyme (ACE) as cause of hypertensive blood pressure by this enzyme. The highest antioxidant activity based on ORAC and TPC was found at 24 h incubation in the co-culture set at 2.58 ± 0.26 µmol TE/ml at 24 h and TPC at 0.31 ± 0.03 µmol GE/ml. In fermented whey, the lowest pH at 4.35 ± 0.01 was observed in a co-culture set with LAB population (log CFU/ml) at 7.19 ± 0.03 for ENM104 and 8.41 ± 0.02 for SPS109. ENM104 set maximally reduced 54.86 ± 13.48 % cholesterol at 72 h. However, GABA content reported in µg/ml in SPS109 set (4.84 ± 0.01) was higher than a co-culture set (4.25 ± 0.03). The highest ACE inhibition at 33.95 ± 4.95 % was found in a co-culture set at 72-h incubation. This set also showed the maximum antioxidant activity both in ORAC and TPC at 0.86 ± 0.22 µmol TE/ml and 0.06 ± 0.01 µmol GE/ml. Overall result prove that both LAB strains have potential to be used as starters for producing functional fermented dairy products.

Keyword: cholesterol, fermented dairy products, functional foods, GABA, lactic acid bacteria, probiotics

ชื่อวิทยานิพนธ์ การแยกและคัดเลือกแลกติกแอซิดแบคทีเรียที่มีคุณสมบัติในการลดคอเลสเตอรอลและผลิตกรดแกมมาอะมิโนบิวไทริกเพื่อการผลิตอาหารเสริมสุขภาพผลิตภัณฑ์นมหมัก

ผู้เขียน นายจิรายุ จิตภักดี

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2562

บทคัดย่อ

ปัจจุบันอาหารเสริมสุขภาพเป็นที่ต้องการของผู้บริโภคมากขึ้น และผลิตภัณฑ์อาหารหมักที่ใช้กล้าเชื้อแลกติกแอซิดแบคทีเรีย (Lactic acid bacteria) ก็เป็นหนึ่งในกลุ่มแนวทางการผลิตอาหารเสริมสุขภาพ ซึ่งงานวิจัยนี้ได้เก็บตัวอย่างอาหารหมักและน้ำนมดิบในประเทศไทยจำนวน 60 ตัวอย่างเพื่อแยกแลกติกแอซิดแบคทีเรียโดยสามารถแยกได้ 244 สายพันธุ์ จากอาหารหมักจำนวน 39 ตัวอย่างและพบว่า 139 สายพันธุ์ผ่านการคัดเลือกระดับปฐมภูมิที่พิจารณาจากการมีชีวิตรอดได้ในอาหาร basal medium ที่เติม 100 µg/ml คอเลสเตอรอล และ 0.1% ยีสต์สกัด แต่มีเพียงจำนวน 10 สายพันธุ์ที่ผ่านการคัดเลือกระดับทุติยภูมิด้วยอาหารชนิดเดียวกันที่ไม่เติมยีสต์สกัดและการคัดเลือกระดับตติยภูมิโดยได้ใช้อาหารชนิดเดียวกันกับการคัดเลือกแบบปฐมภูมิแต่พิจารณาการเจริญของเชื้อหลังจากการบ่ม 72 ชั่วโมง พบว่ามีเพียง 5 สายพันธุ์ได้แก่ ENM104, ENM203, ENM204, NNM205 และ NNM304 จากทั้ง 5 สายพันธุ์นี้พบว่ามีเพียงแค่สายพันธุ์ ENM104 ที่มีความสามารถลดปริมาณคอเลสเตอรอลได้มากที่สุดในอาหาร basal medium ที่เติม 100 µg/ml cholesterol-PEG600 โดยลดได้ 7.53 ± 1.78 % ที่การบ่ม 72 ชั่วโมง สำหรับการคัดเลือก LAB ที่สามารถผลิตสารกาบาได้จากจำนวน 10 สายพันธุ์ที่ผ่านการคัดเลือกจากระดับทุติยภูมิมาทดสอบในอาหาร MRS ที่เติม 5 mg/ml monosodium glutamate (MSG) พบว่ามีเพียงสายพันธุ์เดียวคือ SPS109 ที่สามารถผลิตได้และผลิตกาบาได้ 1,157 ± 4.76 µg/ml ที่การบ่ม 72 ชั่วโมง การเทียบเคียงสายพันธุ์ของ LAB ที่ผ่านการคัดเลือกได้ทดสอบทั้งวิธีทางชีวเคมีด้วยการหมักน้ำตาล 49 ชนิดใช้ชุดทดสอบ API 50 CHL และทางอณูชีววิทยาด้วยการตรวจสอบยีน 16S rRNA พบว่าสายพันธุ์ ENM104 มีความใกล้เคียงกับ *Pediococcus pentosaceus* DSM20336^{T} ที่ 99.65 % และสายพันธุ์ SPS109 มีความใกล้เคียงกับ *Lactobacillus plantarum* DSM20174^{T} ที่ 99.93 % การทดสอบความสามารถการเป็นโปรไบโอติกพบว่าทั้งสองสายพันธุ์ไม่สามารถย่อยสลายแป้ง ขณะที่สายพันธุ์ SPS109 สามารถย่อยสลายไตรบิวไทริน แต่ทั้งสองสายพันธุ์สามารถย่อยสลายเคซีน ในส่วนของการย่อยสลายกรดน้ำดีพบว่าทั้งสองสายพันธุ์สามารถย่อยสลาย glycocholic acid, taurocholic acid และ taurodeoxycholic acid ได้ ในส่วนของการทดสอบการทนทานต่อระบบทางเดินอาหารแบบจำลองพบว่าทั้งสองสายพันธุ์สามารถทนต่อสภาวะกรดที่จำลองจากกระเพาะอาหารได้โดยตรวจพบเซลล์จำนวน 6.20 ± 0.12 และ 7.39 ± 0.12 log CFU/ml จากเซลล์ตั้งต้น 10.18 ± 0.02 และ 10.46 ±

0.04 log CFU/ml ของสายพันธุ์ ENM104 และ SPS109 ตามลำดับ นอกจากนี้จำนวนเซลล์เพิ่มขึ้นเล็กน้อยหลังผ่านเข้าสู่สภาวะเบสที่จำลองจากลำไส้เล็กที่ 6.49 ± 0.00 และ 7.63 ± 0.02 log CFU/ml ส่วนคุณสมบัติการลดปริมาณคอเลสเตอรอลในอาหาร MRS ที่เติม 100 µg/ml คอเลสเตอรอล พบว่าสายพันธุ์ ENM104 และ SPS109 ลดคอเลสเตอรอลได้ 15.34 ± 1.12 และ 10.05 ± 2.32 % ตามลำดับที่การบ่ม 72 ชั่วโมงของ ซึ่งสายพันธุ์ ENM104 สามารถผลิตเอนไซม์คอเลสเตอรอลออกซิเดสโดยมีกิจกรรมจำเพาะของเอนไซม์ที่ 8.73 ± 2.00 mU/mg protein เมื่อบ่ม 72 ชั่วโมงในอาหาร MRS ที่เติม 100 ug/ml cholesterol-PEG600 นอกจากนี้ได้มีการตรวจวัดการทนต่อไฮโดรเจนเปอร์ออกไซด์ความเข้มข้น 1 mM พบว่าสายพันธุ์ ENM104 สามารถทนได้ 20 ชั่วโมงและสายพันธุ์ SPS109 สามารถทนได้ 12 ชั่วโมง รวมถึงได้มีการตรวจวัดการต้านอนุมูลอิสระ ORAC ของเชื้อในอาหาร MRS พบว่าสายพันธุ์ ENM104 ให้ผลสูงสุดที่ 48 ชั่วโมงที่ 37.26 ± 0.77 µmol TE/ml และสายพันธุ์ SPS109 ให้ผลสูงสุดที่ชั่วโมงที่ 72 ที่ 34.01 ± 3.30 µmol TE/ml ส่วนการตรวจวัดปริมาณสารฟินอลิกทั้งหมดพบว่าให้ค่าไม่ต่างกันที่ 0.67 และ 0.68 µmol GE/ml ตามลำดับ ในการประยุกต์ทำนมหมักและเวย์หมักด้วยการใช้กล้าเชื้อเดี่ยวและกล้าเชื้อผสม (อัตราส่วน 1:1) พบว่าในนมหมักให้ค่า pH ต่ำสุดที่ 5.32 ± 0.01 ของชุดกล้าเชื้อผสมที่การบ่ม 72 ชั่วโมงซึ่งให้จำนวนเชื้อของสายพันธุ์ ENM104 ที่ 6.93 ± 0.01 และของสายพันธุ์ SPS109 ที่ 8.27 ± 0.04 log CFU/ml และลดปริมาณคอเลสเตอรอลในนมหมักได้ 15.71 ± 0.64 และ 10.98 ± 3.80 % ในชุด ENM104 และชุดกล้าเชื้อผสมของนมหมักที่การบ่ม 72 ชั่วโมง ส่วนการผลิตสารกาบาในชุด SPS109 และชุดกล้าเชื้อผสมพบว่าให้ผลที่ 4.57 ± 0.01 และ 4.47 ± 0.04 µg/ml ตามลำดับ ส่วนของการผลิตสารยับยั้งการทำงานของเอนไซม์ angiotensin-converting enzyme (ACE) ที่ช่วยในการลดภาวะความดันโลหิตสูงของชุดหมักแบบเชื้อผสมให้ผลการยับยั้งที่ 57.63 ± 2.97 % นอกจากนี้พบว่าการผลิตสารต้านอนุมูลอิสระชุดเชื้อผสมที่การบ่ม 24 ชั่วโมงให้ผลสูงสุดทั้ง ORAC ที่ 2.58 ± 0.26 µmol TE/ml และปริมาณสารฟินอลิกทั้งหมดที่ 0.31 ± 0.03 µmol GE/ml ในส่วนของเวย์หมัก ชุดเชื้อผสมให้ผลค่า pH ต่ำสุดที่ 4.35 ± 0.01 ซึ่งนับการเจริญของสายพันธุ์ ENM104 และ SPS109 ได้ 7.19 ± 0.03 และ 8.41 ± 0.02 log CFU/ml ตามลำดับ ส่วนการลดปริมาณคอเลสเตอรอลในเวย์หมักชุดกล้าเชื้อ ENM104 ที่ 72 ชั่วโมงพบว่าสามารถลดได้สูงสุด 54.86 ± 13.48 % ส่วนการผลิตสารกาบาพบว่าชุด SPS109 และชุดกล้าเชื้อผสมผลิตได้ 4.84 ± 0.01 และ 4.25 ± 0.03 % ตามลำดับ ส่วนการผลิตสารยับยั้ง ACE พบว่าชุดกล้าเชื้อผสมให้ผลการยับยั้งสูงสุดที่ 33.95 ± 4.95 % และผลิตสารต้านอนุมูลอิสระวิธี ORAC ให้ผลสูงสุดที่ 0.86 ± 0.22 µmol TE/ml และพบปริมาณสารฟินอลิกทั้งหมดสูงสุดที่ 0.06 ± 0.01 µmol GE/ml ดังนั้นจากผลการศึกษาพบว่าการใช้กล้าเชื้อผสมทั้งสองชนิดมีศักยภาพในการใช้ผลิตนมหมักและเวย์หมักเป็นอาหารเสริมสุขภาพได้

คำสำคัญ: กาบา, คอเลสเตอรอล, ผลิตภัณฑ์นมหมัก, โปรไบโอติก, แลกติกแอซิดแบคทีเรีย, อาหารเสริมสุขภาพ

ACKNOWLEDGEMENTS

I would like to thank the important source of funds by the Faculty of Science Research Fund of Prince of Songkla University (PSU) with contract no. 1-2559-02-016 and partial scholarship support from Graduate School, PSU. To thank many personnel, I would like to respect the advisor of this thesis named Prof. Dr. Duangporn Kantachote to support, consult and plan this thesis to be success. Moreover, I would like to thank several instructors to support and consult including Prof. Dr. Supayang Voravuthikunchai, Assoc. Prof. Dr. Pharanai Sukhumungoon, Asst. Prof. Dr. Piyarat Sirivongpaisal, Asst. Prof. Dr. Ladda Leelawatwattana and Dr. Onnicha Rattanaporn. Besides, I would like to thank committees for honorary time to advocate of thesis defence including Assoc. Prof. Dr. Preeyanuch Bovornreungroj, Asst. Prof. Dr. Sasithorn Sirilun and the advisor.

For the valuable experience (9 July 2018 – 23 July 2018), I would like to salute Asst. Prof. Dr. Chaiyavat Chaiyasut, and Dr. Wattana Pelyuntha at the faculty of Pharmacy, Chiangmai University. For overseas internship experience (1 July 2019 – 30 September 2019), I would like to thank Japan Student Services Organization (JASSO) scholarship with registrant number WSA1910700301007 to support the life expenditure and thank to Prof. Hiroshi Kanzaki and Assoc. Prof. Dr. Teruhiko Nitoda with important student named Atsuko Hashimoto of Graduate School of Environmental and Life Science, Faculty of Agriculture, Okayama University to teach some special techniques.

To focus to the persons to importantly associate to discover fermented foods and raw milk samples located in various regions of Thailand, there was included the name lists representing Angkana Thungkaew, Chanida Saekow, Chatchada Pankling, Katika Maneenin, Manutsavee Manomuttaya, Marium Bissanum, Patama Bunruk, Prapada Jakkarn, Rujira Tantadapan, Saifah Parasi and Varee Romsakul.

Finally, I would like to thank my family to back up for master's degree learning with some helpful friends of ST608 room and overseas internship students to collaborate in our laboratory and teach some worthy techniques named Chifumi Shinagawa from Ishikawa Prefectural University, Komaki Kurihara and Rii Hatamoto from Okayama University, Japan.

Jirayu Jitpakdee

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LIST OF ABBREVIATIONS AND SYMBOLS

ACE	=	angiotensin-converting enzyme
AD	=	4-androstenedione
ADD	=	1,4-androstenedione
BOD	=	biological oxygen demand
BSH	=	bile salt hydrolase
ChoX	=	cholesterol oxidase
CLA	=	conjugated linoleic acid
COD	=	chemical oxygen demand
CVD	=	cardiovascular disease
EDTA	=	ethylenediaminetetraacetic acid
FAD	=	flavin adenine dinucleotide
GABA	=	γ-aminobutyric acid
GAD	=	glutamic acid decarboxylase
GI	=	gastrointestinal
GMC	=	glucose-methanol-choline
GRAS	=	generally recognised as safe
HDL	=	high-density lipoprotein
HMG-CoA	=	β-hydroxy-β-methylglutaryl-CoA
HPLC	=	high-performance liquid chromatography
J/kg·K	=	Joule per kilogramme Calvin
LAB	=	lactic acid bacteria
LDL	=	low-density lipoprotein
mN/m	=	millinewton per metre
MRS	=	de Man Rogosa and Sharpe
MSG	=	monosodium glutamate
MW	=	molecular weight
osmol/kg	=	osmole per kilogramme
R_f	=	retardation factor
S/m	=	Siemen per metre
TLC	=	thin layer chromatography
W/m·K	=	Watt per metre Calvin

CHAPTER 1

INTRODUCTION

1. Background and rationale

In the today's world, humanity suffers from various diseases and illnesses to be severe to either morbidity or mortality. Cardiovascular disease (CVD) is one of several diseases with the estimated mortality rate for 17.9 million people annually worldwide (WHO 2020), particularly the elderly and obese. Moreover, it has estimated that the rate in the year 2030 should be around 23.6 million people globally per year (Cannon 2013).

Human's behaviour consumptions from social atmospheres are caused to activate the imbalance of lipid transportation. One factor to be related is cholesterol content in diets. Cholesterol is categorised as one of the lipids and can establish in the liver. This substance is transported in form of lipoprotein especially low-density lipoprotein (LDL) (Nelson and Cox 2008) to communicate between liver as synthesis station and adipose tissues combined with muscle cells for storage location. Bulk consumption of cholesterol can disturb this body's storage system to generate the oversupply of LDL in bloodstream. Then this lipoprotein has high incident to itself agglutination and subsequently, attaches to the artery wall for generating the fatty-clogged formation named atherosclerosis which is one of the CVD.

To decline the incident of this atherosclerosis, the consumption of low cholesterol diets provided by cholesterol-lowering lactic acid bacteria (LAB) is one of alternative ways for the solution to relieve. These bacteria are famous as "Generally Recognised As Safe (GRAS)" (Narvhus and Axelsson 2003) that it is safe to use as food additives to perform the functional foods for consumer's health improvement expectation. Furthermore, some LAB strains can be probiotic candidates based on the principle "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" determined by FAO/WHO (Morelli and Capurso 2012).

Besides, LAB strains provide other valuable functions to support the advantageous aspects, particularly the improvement of the consumer's health. For

example, they produce and release γ-aminobutyric acid (GABA) as a function to convert glutamic acid to GABA by glutamic acid decarboxylase (GAD). Numerous functions of this compound for human's health include of the action of neurotransmitter as primary inhibitor of nervous system in sympathetic part, low bloodstream pressure, urine secretion and anti-depressing disorder (Wong et al. 2003). Most of LAB strains have been applied to add value in fermented food i.e. *Pediococcus pentosaceus* HN8 and *Lactobacillus namurensis* NH2 were used as starters for producing Thai fermented pork sausage (Nham) that enriched with GABA, but reduced cholesterol content (Ratanaburee et al. 2013a). Other functional activities related to anti-hypertensive action of bloodstream is the performing of peptides which are called either casokinin or lactokinin to obstruct the renin-angiotensin mechanism called angiotensin-converting enzyme (ACE) inhibitor, and these peptides were reported only in fermented dairy products (Mozzi et al. 2010). Some strains of *Lactobacillus*, *Weissella* and *Lactococcus* were reported for their abilities to produce ACE inhibitor (Barla et al. 2016; Hagi et al. 2016). Besides, several researchers have reported LAB significantly increased antioxidant activity in fermented foods and beverages (Abubakr et al. 2012; Agbor et al. 2014; Kantachote et al. 2017; Kudoh et al. 2001) with some productions of bioactive peptides in fermented dairy products.

With the whole advantageous aspects of those LAB activities, the applications to produce the functional foods and beverages have been explored with the expectation to improve the consumer's health. The abundant diet to be suggested to cover all functions and the most simplified diets is dairy products with the contribution of both milk and whey. Milk production worldwide has increased over the decade from 702 in 2009 to 852 million tonnes in 2019 (FAO 2012, 2019). Production of milk products can refer to the increase of massive consumption owning to the consideration of valuable nutrients include by carbohydrate, protein particularly casein as main portion, fat by which cholesterol is composed, vitamins and trace elements. However, by-product from dairy product industry like liquid whey is unavoidable threatened to ecosystem. Whey is the disposal part as an environmental problem with biological oxygen demand (BOD) around 32,000 to 60,000 mg/L equally level of 60,000 to 80,000 mg/L for chemical oxygen demand (COD). As high content of lactose in whey, the usage of this by-product is difficult (Cheryan 2000).

Regarding above information, it would be possible that potent LAB can be used as starters for producing milk and whey to be functional foods for reducing of cholesterol, while fortifying with GABA as the neurotransmitter, ACE inhibitor for reflecting to an anti-hypertensive agent and antioxidative compounds to face with free radicals in consumer's host. Moreover, the use of whey to produce fermented dairy product beverage containing with vital substances by potent LAB fermentation is the way out to solve the environmental problem and respond the alternative product. Hence, this thesis focused on isolation and selection of promising LAB based on reducing cholesterol content, while producing GABA, ACE inhibitor including antioxidants for using as starters in fermented milk and fermented whey.

2. Objectives

1. To isolate and select LAB from fermented foods and raw milk with cholesterol-lowering and GABA-producing activity,
2. To evaluate the number of cholesterol reduction by selected LAB in synthetic medium and enzyme activity from cholesterol degradation,
3. To identify selected LAB using phenotypic and genotypic characteristics,
4. To investigate probiotic properties of selected LAB based on biological barriers and their benefits, and
5. To apply selected LAB as starters for producing functional milk and whey.

3. Scope of the study

This study focussed to isolate LAB from fermented foods and raw milk and then screen with two characteristics including cholesterol-lowering and GABA-producing activity. Thus, selected LAB strains were investigated for cholesterol oxidase enzyme activity to degrade cholesterol. All selected LAB strains were identified using conventional method and 16S rRNA genes. Probiotic properties based on biological barrier and benefits of selected LAB were explored. In the application part, selected probiotic LAB strains were used as starter culture(s) to produce functional dairy products including milk and whey with aspects to reduce cholesterol, but enriched with GABA, ACE inhibition and antioxidant activity.

4. Expected outcomes

1. To obtain cholesterol-lowering LAB with the activity of cholesterol degradation by cholesterol oxidase,
2. To obtain GABA-producing LAB isolated from fermented foods and raw milk,
3. To provide more information of selected LAB for their probiotic properties including benefits, and
4. To apply selected LAB as starters for producing functional dairy products.

Regarding all above outcomes, it should be possible for commercial production of functional fermented dairy products with achievement of cholesterol-lowering content, while enhancing of GABA, ACE inhibitors, and antioxidants. However, the sensory evaluation of the functional fermented dairy products should be investigated before enlarging to commercial scale.

CHAPTER 2

LITERATURE REVIEW

1. Cardiovascular disease (CVD)

Cardiovascular disease (CVD) or coronary artery disease (CAD) is a world's citizen problem to be faced with the estimated mortality rate for 17.9 million people annually-globally (WHO 2020), approximately around 31 percent of all mortalities and forecasted the number in the year 2030 around 23.6 million people worldwide (Cannon 2013). Major cause for CVD is ischaemic heart disease related to non-supply blood to coronary artery that causes from atherosclerosis to clog arteries from fatty particles. Factors to activate this clogged disease are composed of fatty supplemented diets, cigarette smoke, problem of body's metabolism, especially in the elderly, etc. The social problems can also activate this disease as well. For example, more high-income citizen usually faces with this disease than the lower income. Endomorphic somatotype's citizen typically encounters over the ectomorphs due to the supplementation and body's metabolisms.

Symptom of this disease is hypertension that 1.13 billion people worldwide are suffered (WHO 2020). Blood pressure is relevant with the flow in arteries across their bodies. Blood pressure can be high or hypertension from some predictable causes such as clogged arteries, coronary problems, and vessel deterioration. Relationship between high cholesterol in blood and heart disease has been published to cause hypertension in CVD. American Health Association declares that the total cholesterol should be lower than 200 mg/dL. Type II diabetes can accelerate lower of high-density lipoprotein (HDL) and higher low-density lipoprotein (LDL) caused to hypertension and atherosclerosis. Moreover, obesity can also be caused by increasing of triglyceride level received from diets that impulse to more LDL production than HDL. For the reasons to describe, the relationship of lipoprotein and cholesterol is given below.

Cholesterol can synthesise in human body, particularly in the hepatic metabolism to provide as biliary cholesterol, bile salts which are more hydrophilic form and cholesteryl ester which is more hydrophobic form preparation of acyl-CoA cholesterol acyl transferase (ACAT) with the combination of fatty acyl CoA. To focus on lipoprotein, this particle is mainly contained in four types including chylomicrons, very-low density lipoprotein (VLDL), LDL and HDL. The component of lipoprotein is comprised of various compositions in difference ratio providing in the Table 2-1. Moreover, the differences of apolipoprotein can appoint those types and their functions as shown in the Table 2-2 for apolipoprotein of the human plasma lipoprotein (Nelson and Cox 2008). Detail description of each type is represented below (Nelson and Cox 2008).

Chylomicrons which are the largest lipoprotein and lowest density composed of the unique apoB-48, apoC-II and apoE. These particles are transported to adipose capillary, heart, skeletal muscle and lactating mammary tissue for storing as a fuel. ApoC-II is activated lipoprotein lipase of the target cell to lyse and gather those lipid molecules. These particles appear after diets were ingested and absorbed by intestine. Excess chylomicrons are sent to liver to lyse with lysosome to cholesterol and triacylglycerols with apoE-receptor-binding protein.

Table 2-1. Type of lipoproteins and compositions (modified from Nelson and Cox, 2008)

Lipoprotein	**Size (nm)**	**Density (g/mL)**	**Composition (%)**				
			Apolipoprotein	**Phospholipid**	**Free cholesterol**	**Cholesteryl ester**	**Triacylglycerols**
Chylomicrons	50-200	<1.006	2	9	1	3	85
VLDL	28-70	0.950-1.006	10	18	7	12	50
LDL	20-25	1.006-1.063	23	20	8	37	10
HDL	8-11	1.063-1.210	55	24	2	15	4

VLDL: very-low density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein

Table 2-2. Apolipoprotein of the human plasma lipoprotein (modified from Nelson and Cox, 2008)

Apo lipoprotein	**Function**	**Lipoprotein**			
		Chylomicrons	**VLDL**	**LDL**	**HDL**
ApoA-I	LCAT activation	×	×	×	●
	ABC transporter interaction				
ApoA-II	LCAT inhibition	×	×	×	●
ApoA-IV	LCAT activation	●	×	×	●
	Clearance and transport of cholesterol				
ApoB-48	Clearance and transport of cholesterol	●	×	×	×
ApoB-100	LDL receptor binding on extrahepatic tissues	×	●	●	×
ApoC-I	*Ambiguous function to be concluded*	×	●	×	●
ApoC-II	Lipoprotein lipase activation	●	●	×	●
ApoC-III	Lipoprotein lipase inhibition	●	●	×	●
ApoD	*Ambiguous function to be concluded*	×	×	×	●
ApoE	Clearance of VLDL & chylomicron remnants	●	●	×	●

Presence (●), absence (×), no information (blank); Abbreviation: LCAT = lecithin-cholesterol acyl transferase

After liver established very-low-density lipoprotein (VLDL) that mostly composes of triacylglycerols, cholesterol and cholesteryl ester, this particle is transported to muscle and adipose tissue as storage fuel by the activity of apoC-II to release the lipid. The excess particles are sent to extrahepatic tissue to store, and the smaller size one is called low-density lipoprotein (LDL) that consists of apoB-100 as VLDL. Moreover, the stored lipid in muscle and adipose tissue is secreted out to send to extrahepatic tissue in the form of LDL. Then this component contains mainly cholesterol and cholesteryl ester over than high-density lipoprotein (HDL). This lipoprotein is secreted out from extrahepatic tissue in the liver part which is performed by protein-rich particle including apoA-I, apoC-I, apoC-II and lecithin-cholesterol acyl

transferase (LCAT) to prevent the cholesteryl ester formation in HDL. Thus, this lipoprotein also comprises of small content of cholesterol with no cholesteryl ester. Some cholesterol from extrahepatic is used as bile acid and biliary cholesterol in the essential volume. Some mechanisms help to decrease to amount of LDL in bloodstream, but it is not enough. Macrophage can catch up LDL in the circulating system with LDL receptor and CD36 receptor located on the cell membrane to uptake into inside the cell. Several regulators transform these particles to HDL (Li and Chiang 2009).

To be concluded, human cell especially hepatocytes can anabolise cholesterol molecule from lipid monomer. Intake of this molecule from elsewhere is not critically essential because cholesterol can be synthesised in various primates. Over-supply of cholesterol in bloodstream which is received from outsources can directly increase the number of LDL particle in blood. Consequently, the congested particle transportation might be generated from the formation of LDL agglutination on the artery vessel to be atherosclerosis causing for CVD. The symptom will be heart failure from the lack of blood circulation. Conversely, HDL rarely affects with the relationship to CVD because cholesterol contains HDL in the tiny volume.

From this problem, various aspects are used to solve to reduce the incident of CVD which causes from high content of cholesterol in bloodstream. Thus, LAB may be one of the alternative aspects to deal with this symptom.

2. Lactic acid bacteria (LAB)

Nowadays, most of LAB strains are popular bacteria to be used as starters in food industry for food production. This bacterial group are not only to act as starters for producing fermented foods, but also can be probiotic candidates to support consumer health. Hence, these bacteria are popular in the food industry to produce functional foods such as dairy products, beverages, sausages, sourdough and so on (Molina et al. 2012).

2.1. General information of lactic acid bacteria (LAB)

Lactic acid bacteria are classified in *Lactobacillaceae* family with the obvious characteristics including Gram's positive with rods or cocci as shape and

diplococci, tetrad, side chain as their bacterial arrangement. No spore production and bacterial movement with flagellum are appeared. Moreover, no catalase enzyme is produced which is used as isolated criteria.

The function of catalase enzyme is neutral oxygen radicals in cells to less radical form. For example, hydrogen peroxide is neutralised by catalase to water and oxygen gas. Then they are not necessary to contain the enzyme in the gene due to growth behaviour that is typically found in microaerobic conditions. To describe, those LAB are not synthesised 'porphyrin' substance including heme which is part of catalase enzyme and cytochrome that drives the electron transport chain for energy generator partway of aerobic conditions (von Wright and Axelsson 2012). Thus, LAB will turn to use fermentation to gain energy by substrate-level phosphorylation, as a key pathway for energy synthesis with the low atmosphere condition to remote from toxic molecule to damage the cell from oxygen.

Lactic acid bacteria are abundant in various fermented foods i.e. dairy products, vegetables, fruits, cereals, and meat. Moreover, humans can be the habitat of these bacteria as found in gastrointestinal and reproductive tracts. For example, *Lactobacillus casei* was isolated from infant faeces. Sugar is used as the main carbon source for fermentation to lactic acid as the product by LAB. Furthermore, the fastidious supplementation needs as growth cofactor to give better growth including vitamins and minerals. Growth condition that relates to oxygen is diverse conditions of aerobic, non-aerobic and microaerobic conditions with the optimum temperature around 30 – 40 °C. Acidic condition is more satisfied by LAB than neutral and basic conditions.

Dominant characteristics to classify LAB out from the other groups are less 50 percent of G+C content in genotypic evaluation. This classification can cut *Bifidobacterium* spp. out from this bacterial group because the G+C content is over than 50 percent that directed to Actinomycetes. However, the popular method to classify LAB is the type of fermentation including homofermentation and heterofermentation (Table 2-3). Differentiation between both fermentative groups is the level of lactic acid production which homofermentation can produce over 80 percent of this acid, while the counterpart gives the result 50 percent of the acid with 20 – 25 percent of carbon dioxide (CO_2) and both acetic acid and ethanol for last 20 – 25 percent.

Table 2-3. Grouping of LAB depending on sugar fermentation (modified from von Wright and Axelsson, 2012)

Sugar type	**Fermentation type**		
	Group I	**Group II**	**Group III**
	Obligately homofermentation	**Facultative heterofermentation**	**Obligately heterofermentation**
Hexose fermentation	+	+	+
Pentose fermentation	-	+	+
FDP aldolase	+	+	-
Phosphoketolase	-	+	+
	Group I lactobacilli	Group II lactobacilli	Group III lactobacilli
		Enterococci	Leuconostocs
		Lactococci	Oenococci
		Pediococci	Weissellas
		Streptococci	
		Tetragenococci	
		Vagococci	

According to long history of LAB found in various fermented foods and beverages, many researchers around worldwide signs for which LAB are not pathogenic bacteria and permit them to 'Generally Recognised As Safe (GRAS)'(Morelli and Capurso 2012). Thus, these bacteria are fashionable to use in food industry to produce functional foods with the beneficial improvement of consumer health. For instance, immunomodulator is generated from the bacterial colonisation in the gastrointestinal tract as the probiotics (Mayo et al. 2010; Wood and Holzapfel 1995; von Wright and Axelsson 2012). Typical grouping of LAB normally categorises with genus including *Aerococcus, Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Oenococcus, Enterococcus and Leuconostoc.* Conventional classification based on each genus provides in the Table 2-5.

To be concluded, nowadays, the categorisation of LAB should be considered various factors altogether including morphological characteristics composed of growth temperature, halotolerance, acid and base tolerances, biochemical characteristics for sugar fermentation, lactic acid structure from the production, chemo-

taxonomical characteristics including cell wall component and fatty acid, and, finally, genotypic information of G+C content and 16S rRNA sequence. Classification in genus level of LAB based on cell shapes and their arrangement is provided as shown in the Table 2-4.

Table 2-4. Genus of LAB (modified from Wood and Holzapfel, 1995; von Wright and Axelsson, 2012)

Genus	**Cell shape**	**Cell arrangement**
Aerococcus	Cocci	Diplococci and tetrad
Carnobacterium	Rod	Diplococci and long chain
Enterococcus	Cocci	Diplococci and short chain
Lactobacillus	Rod	Diplococci and long chain
Lactococcus	Cocci	Diplococci and long chain
Leuconostoc	Coccobacilli	Long chain
Oenococcus	Cocci	Long chain
Pediococcus	Cocci	Diplococci and tetrad
Streptococcus	Cocci	Diplococci and long chain
Tetragenococcus	Cocci	Diplococci and tetrad
Vagococcus	Cocci	Non-arrangement
Weissella	Cocci and rod	Long chain

2.2. Grouping of LAB strains depending on sugar fermentation

Classification of LAB on the basis of sugar fermentation types can be divided to homofermentative LAB for using glycolytic pathway or, another name, Embden-Meyerhof-Parnas pathway (EMP) with only lactic acid as the final product and heterofermentative LAB using 6-phosphogluconate-phosphoketolase pathway for sugar catabolism with lactic acid, ethanol, acetic acid and carbon dioxide as the final products. However, lactobacilli in LAB can be categorised into three groups based on sugar fermentation (Table 2-6) (von Wright and Axelsson 2012).

Table 2-5. Differentiation of LAB based on conventional classification (modified from Mayo et al., 2010)

Characteristic	**Rods**		**Cocci**							
	Carnobacterium	***Lactobacillus***	***Aerococcus***	***Enterococcus***	***Lactococcus Vagococcus***	***Leuconostoc Oenococcus***	***Pediococcus***	***Streptococcus***	***Tetragenococcus***	***Weissella*** [a]
Tetrad	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose [b]	-[c]	±	-	-	-	+	-	-	-	+
10 ºC growth	+	±	+	+	+	+	±	-	+	+
45 ºC growth	-	±	-	+	-	-	±	±	-	-
6.5% NaCl growth	ND[d]	±	+	+	-	±	±	-	+	±
18% NaCl growth	-	-	-	-	-	-	-	-	+	-
pH 4.4 growth	ND	±	-	+	±	±	+	-	-	±
pH 9.6 growth	-	-	+	+	-	-	-	-	+	-
Lactic acid type	L	D, L, DL[e]	L	L	L	D	L, DL[e]	L	L	D, DL[e]

Symbols: Positive (+), negative (-), depended on species (±), No data (ND)

[a] Probable result to rod shape in *Weissella* spp.

[b] Glucose fermentation: homofermentative (-), heterofermentative (+)

[c] Slight CO_2 production depending on carbon sources

[d] No report of 8% NaCl growth

[e] D-, L-, DL- lactic acid production

Group I, namely obligate homofermentation is the group to ferment sugar by only glycolytic pathway using FDP aldolase as a key enzyme because no phosphoketolase enzyme to use sugars with five carbons. Thus, this group cannot use five carbon sugars as the carbon source for fermentation.

Group II, namely facultative heterofermentation is the group to have both homolactic and heterolactic fermentations. The point of difference from the group I is the existing of both phosphoketolase enzyme to consume five carbon sugars and FDP aldolase enzyme to utilise six carbon sugars by glycolytic pathway.

Group III, namely obligate heterofermentation is the group to ferment by only 6-phosphogluconate-phosphoketolase pathway due to the containing of only phosphoketolase. Thus, this group cannot ferment by homolactic fermentation.

Table 2-6. Biochemical groups of lactobacilli (modified from von Wright and Axelsson, 2012)

Characteristics	**Group I: Obligate homofermentation**	**Group II: Facultative heterofermentation**	**Group III: Obligate heterofermentation**
Pentose fermentation	-	+	-
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
FDP aldolase	+	+	-
phosphoketolase	-	+[b]	+
	Lb. acidophilus	*Lb. casei*	*Lb. brevis*
	Lb. delbrueckii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantarum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sakei*	*Lb. reuteri*

[a] Only fermentation state

[b] Using pentose as substrate

2.3. Metabolism of lactic acid bacteria

2.3.1. Hexose fermentation

Hexose is the sugar containing six carbon atoms such as glucose, mannose, galactose, fructose and so on. Hexose fermentation catabolises those sugars to the micro-molecules and energy outcome (von Wright and Axelsson 2012). To describe glycolysis or Embden-Meyerhof-Parnas (EMP) pathway in the Figure 2-1, glucose enters to inside of cell with phosphotransferase system (PTS) in glucose-6-phosphate form by which phosphoenolpyruvate (PEP) is the metabolite to control rate of reaction of PTS. Besides, glucose can be entered by permease enzyme, ATP-binding cassette transporter and glycoside-pentoside-hexuronide transporter. When glucose enters to glycolytic pathway for homolactic fermentation, ATP energy is used for

glucose to fructose-1,6-diphosphate (FDP). After the conversion of FDP to glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) by fructose-1,6-diphosphate aldolase (FDP aldolase) that is the key enzyme to control the whole glycolytic pathway rate of reaction, and this enzyme is also used to separate between homolactic and heterolactic fermentations, DHAP can convert to G3P by isomerase enzyme. Subsequently, G3P releases NADH + H^+ and ATP as the high energy compounds until the reaction will terminate to obtain pyruvate. This pyruvate can be fermented to lactic acid by NAD^+-dependent lactate dehydrogenase using NADH + H^+ as the power of reducing agents. Finally, the outcome energy from this catabolism net receives for two molecules of ATP and one molecule of lactic acid.

For heterolactic fermentation that uses 6-phosphogluconate-phosphoketolase pathway, glucose changes to 6-phosphogluconate by dehydrogenation before entering to phosphoketolase which is the key enzyme of this pathway to control the conversion of six to five carbon sugars by decarboxylation. Phosphoketolase transforms xylulose-5-phosphate to G3P which enters to triose phosphate pathway like lower glycolytic pathway and, the other, acetyl-phosphate. Acetyl-phosphate is oxidised by NADH + H^+ for converting to ethanol as the product. Then the outcome from this pathway is composed of one molecule of ethanol, carbon dioxide and ATP.

Galactose is the hexose monosaccharide which has a cell-entering pattern to differ from glucose that can be joined in two types including phosphotransferase and permease. The PTS allows the galactose in form of galactose-6-phosphate before changing to tagatose-1,6-diphosphate and being degraded by aldolase enzyme to DHAP and G3P. This process can be called tagatose-1,6-phosphate pathway. Another sugar entrance is permease. Galactose is converted to galactose-1-phospahte before glucose-1-phosphate conversion and to join to glycolytic pathway in advance. This pathway can be called Leloir pathway in the Figure 2-2.

2.3.2. Disaccharide fermentation

Disaccharide fermentation is described about the usage of two-molecule-conjugated sugar such as sucrose, lactose, maltose and so on to catabolise to micro-molecules and outcome energy. Disaccharide enters the cell in the form of disaccharide-phosphate before degrading of phosphohydrolase enzyme to monosaccharide and monosaccharide-phosphate. For example, lactose phosphotransferase (PTS) found in *Lactobacillus casei* to take lactose into inside cell and change to lactose-phosphate before hydrolysing of phospho-β-D-galactosidase to glucose and galactose-phosphate, and then glucose entering to glycolytic pathway, while galactose-phosphate is reacted by tagatose-6-phosphate pathway (Figure 2-2). Another instance is explained for lactose permease in *Lactococcus lactis* for bringing lactose to inside cell before utilising by β-galactosidase to glucose and galactose. Some strains of LAB after consuming lactose is managed, galactose is excreted out of the cell such as *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus acidophilus*. Likewise, maltose is found as similar above mechanism by maltose phosphorylase enzyme to cut maltose to glucose and β-glucose-1-phosphate that the last substance can be used as one component of cell wall. For sucrose, this sugar enters to inside the cell by sucrose hydrolase and then is hydrolysed to glucose-6-phsophate and fructose (von Wright and Axelsson 2012).

2.3.3. Pentose fermentation

When pentose enters to cell pathway, it is converted by epimerase or isomerase enzyme to ribulose-5-phosphate or xylulose-5-phosphate. Subsequently, this conversion is joined with 6-phosphogluconate or phosphoketolase pathway for sugar fermentation. This activity can be found in both homolactic and heterolactic fermentations, excepted Group I: lactobacilli for obligately homofermentation, and this fermentation is not discovered any carbon dioxide production, so no exceeded carbon is eliminated (von Wright and Axelsson 2012).

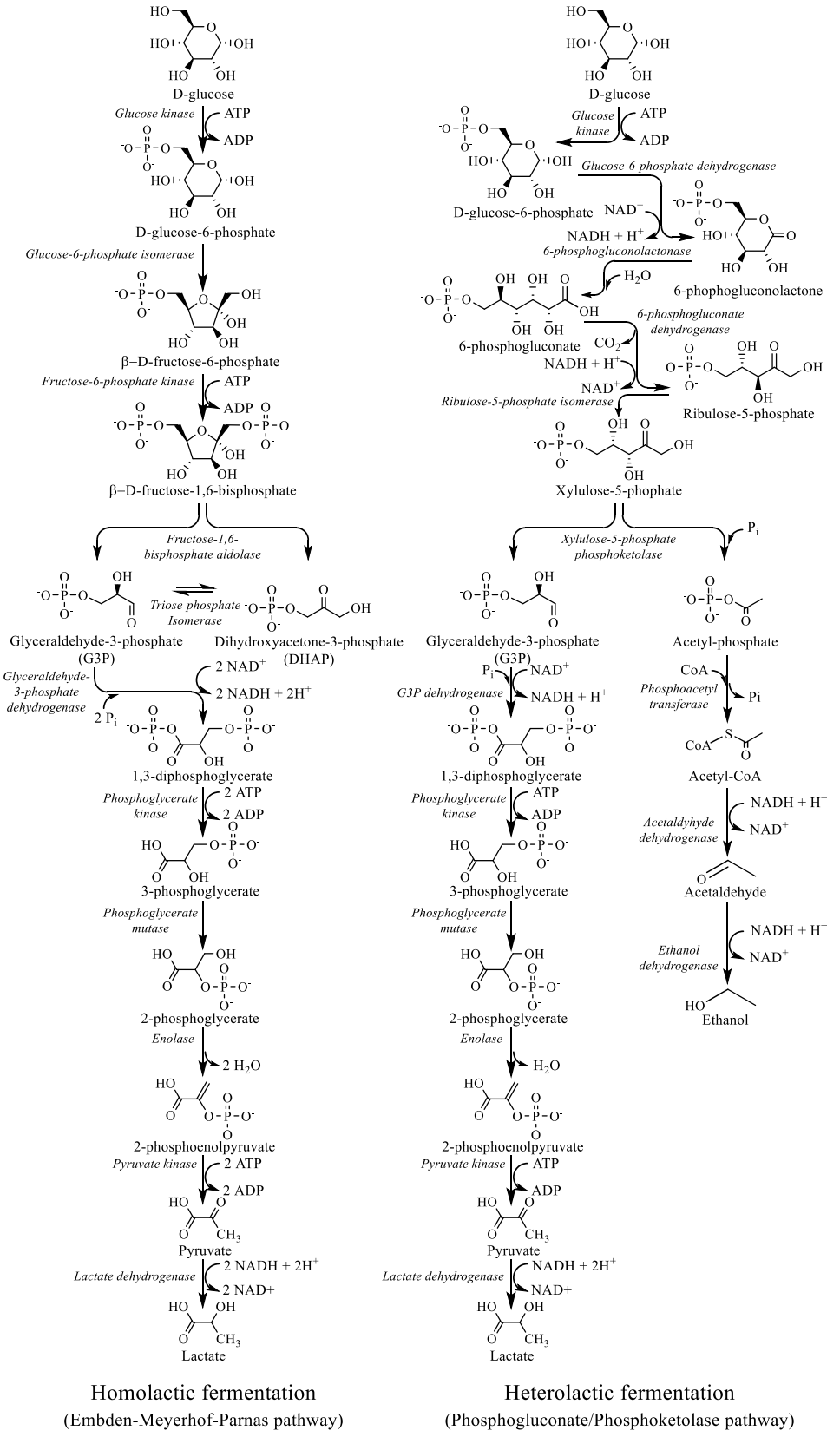


Figure 2-1. Pathways of glucose fermentation: homolactic and heterolactic fermentations (modified from von Wright and Axelsson, 2012)

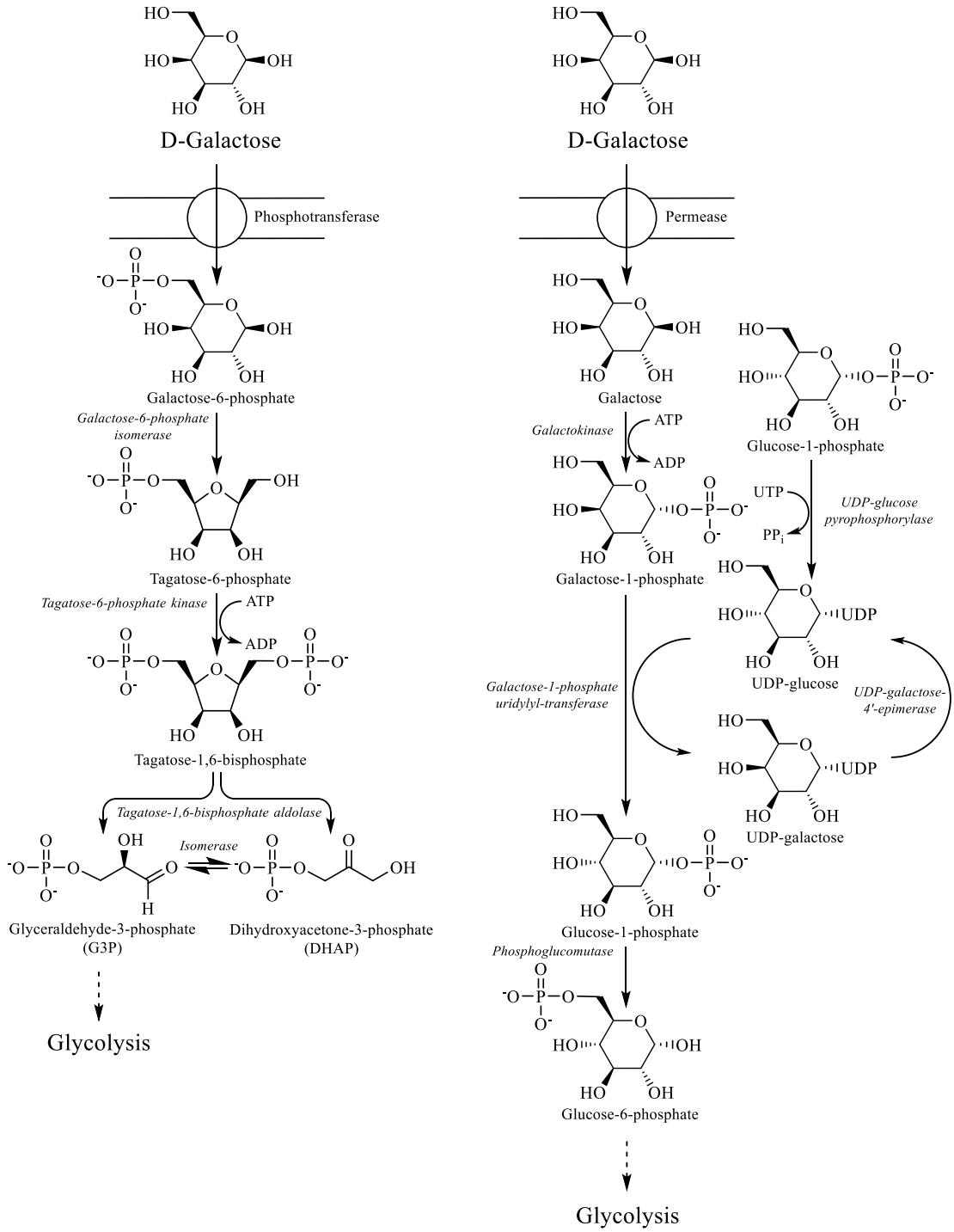


Figure 2-2. Pathway of galactose fermentation: tagatose- 6- phosphate and Leloir pathway (modified from von Wright and Axelsson, 2012)

2.3.4. Transformation of lactic acid molecule structure

LAB can produce lactic acid into 2 isomers including D- and L-lactic acid depending on type of enzyme establishment composed of D- and L-lactate dehydrogenase in which both enzymes can be found each or both enzymes in one bacterial strain. Besides, some strains of LAB found only one type of enzyme can be discovered for 2 types of lactic acid owning to the existing of racemase enzyme to convert between these stereoisomers (von Wright and Axelsson 2012).

2.3.5. Conversion of pyruvate to acetoin, diacetyl and 2,3-butanediol

Figure 2-3 shows *Lactococcus lactis* holding the activity to change pyruvate to diacetyl as aroma for butter industry in low sugar-containing and high acidic condition. Pyruvate is renovated by acetolactate synthase to α-acetolactate in acidic-aerobic conditions before oxygen is taken to that molecule to diacetyl and carbon dioxide. Besides, the intermediate can be released carbon dioxide out by α-acetoin decarboxylase to acetoin. Then acetoin can continue with acetoin reductase to terminate to 2,3-butanediol as the end product (von Wright and Axelsson 2012).

2.3.6. Conversion of pyruvate to acetate by pyruvate oxidase system

Under aerobic condition, pyruvate is converted in the aerobic condition to acetyl-phosphate with hydrogen peroxide as by-product generation, and this reaction is terminated to acetic acid as the final product. This partway found in some strains of *L. plantarum* as described in the Figure 2-3 (von Wright and Axelsson 2012).

2.3.7. Conversion of pyruvate by pyruvate-formate lyase system and pyruvate dehydrogenase system

Pyruvate-formate lyse system is opposite from pyruvate dehydrogenase system owning to the anaerobic-conditioning generation. Pyruvate is transformed to formate and acetyl-CoA by pyruvate-formate lyase system and the co-operation of coenzyme A. Conversely, pyruvate dehydrogenase system is changed the pyruvate by reducing of NAD^+ to $NADH^+ H^+$ with the elimination of carbon dioxide to terminate to

acetyl-CoA. Both pathways (Figure 2-3) are switched work depending on aerobic conditions (von Wright and Axelsson 2012).

Many edible bacteria are categorised in LAB; however, host improvement and supplementation are better aspects whether those LAB are probiotic strains as well. Then the next topic reviews for explanation about the qualification of probiotic strains.

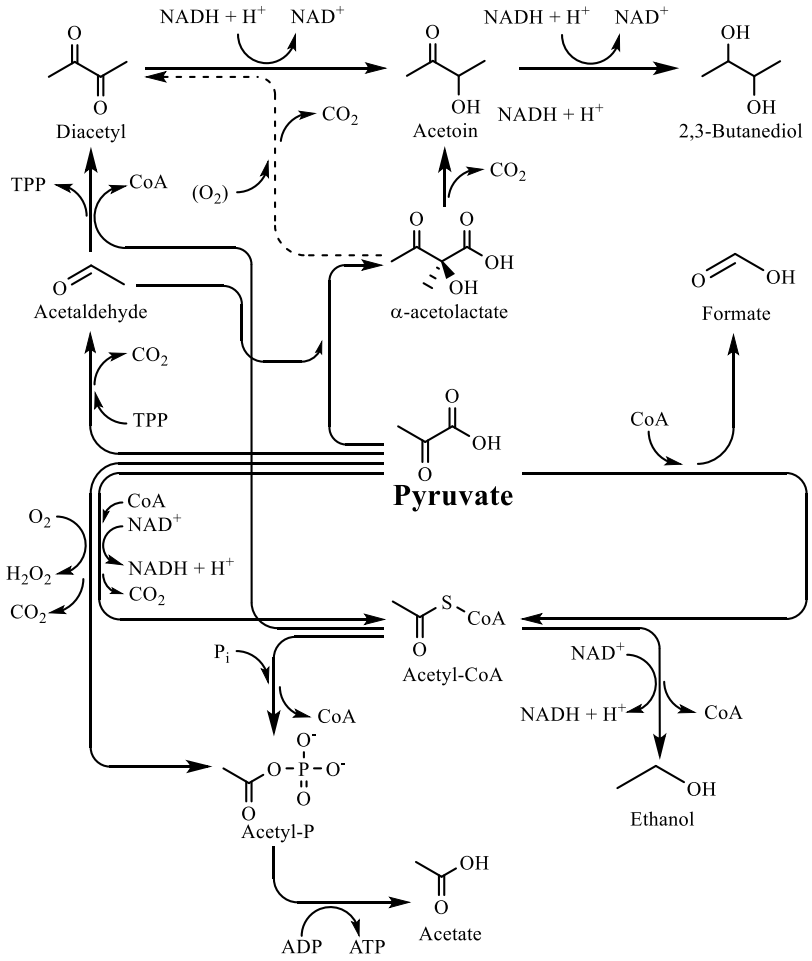


Figure 2-3. Citrate metabolism (modified from von Wright and Axelsson, 2012).

3. Probiotics

FAO/WHO defines probiotics as “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (Morelli and Capurso 2012). Most probiotics are belonged to LAB with have been known as “Generally Recognised As Safe (GRAS)” by FDA in the united states and “Qualified Presumption of Safety (QPS)” by European nation (Narvhus and Axelsson 2003).

Probiotic candidates should be tested the potential for stability to supply in diets including control of pathogenic colonisation, regulation of immunised system, relief of lactose dysfunction, cholesterol-lowering activity, dealing with large intestinal cancer and improvement of nutrient absorption. To start with the control of pathogenic colonisation, probiotic should be activated to have the prophylaxis amongst pathogenic threatening with the replacing by probiotic colonisation by competitive elimination, nutrient competition, activation of immunomodulation and some antimicrobial productions such as organic acids, bacteriocins and hydrogen peroxide tolerance. For regulation of immunised system, this topic is related to host immune system. Several reports are displayed probiotics and brain communication which result for inflammatory of gastrointestinal illnesses, obesity, and consuming disorders. Likewise, stress can increase the permeability of cell in intestinal tract relating mast cell activation and interferon-γ production, and some evidences are found for controlling of mitosis and apoptosis balance (Nuñez et al. 2015).

For relief of lactose dysfunction, human’s body who is absent for lactose utilisation by β-galactosidase. Thus, probiotic trait should function for lactose digestion by containing of β-galactosidase to digest that sugar for host absorption in advance. By the way, the cholesterol-lowering activity should be contained in probiotic candidates for reducing cholesterol accumulation from ingested diets to prohibit cholesterol adsorption to intestinal cell. Moreover, the deconjugated function for bile salts should be discovered to hydrolyse bile salt molecules. These deconjugated bile salts cannot be reabsorbed to circulating system due to the dysfunction, but it is contaminated to faeces. For dealing with large intestinal cancer, probiotics should produce some compounds to reduce the proliferation of tumour cells. Some reports show three effective enzymes to be repressed including β-glucuronidase, azoreductase and nitroreductase which have

the relationship with carcinogen performing in gastrointestinal (GI) tract. Besides, the indispensable factor to contain for probiotic traits comprises of normal population of intestine, host specificity, acid and bile salt toleration, alive during transportation and production of beneficial substance for health improvement (Gilliland 2003).

For cholesterol-lowering property in probiotic candidates, mechanisms to deal with cholesterol both in diets and consumer's body consist of five aspects. Firstly, after colonisation of probiotics on the intestinal cell, they can bind and assimilate the cholesterol from the diets (Gilliland et al. 1985; Pereira and Gibson 2002; Tomaro-Duchesneau et al. 2014; Tsai et al. 2014). This mechanism can prevent the absorption of cholesterol by epithelial intestinal cell. Secondly, the activity of bile salt deconjugation by probiotics can also prevent. Various promising probiotic bacteria should be contained this activity to cancel the cholesterol input to systemic circulation and secret out with the faeces (Erkkilä and Petäjä 2000; Pennacchia et al. 2004; Ratanaburee et al. 2013b; Tanaka et al. 1999). Thirdly, the production of conjugated linoleic acid (CLA) especially in *Lactobacillus* and *Streptococcus* is found to have a relationship to reduce HDL/LDL ratio (Gorissen et al. 2011; Pessione 2012). Fourthly, the production of ferulic acid by *Lactobacillus fermentum* NCIMB5221 can interfere β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase in cholesterol synthesis (Tomaro-Duchesneau et al. 2012). Lastly, the conversion of cholesterol into other metabolites should be possible. For example, the conversion to coprostanol by *Lactobacillus* spp. to reduce the cholesterol absorption by the epithelial cells (Lye et al. 2010). As the persistence of this compound, so it can be found in faeces. Another example is the conversion to 4-cholesten-3-one by *Lactobacillus helveticus* CD6 that can reduce the epithelial absorption as well (Ahire et al. 2012).

To be known to prospective bacteria, tools of identification should be studied to be classified those probiotic candidates and provided the nomenclature in advance.

4. Identification, classification, and nomenclature

Relationship amongst other microorganisms is indicated by the identification, classification and nomenclature based on the theory of taxonomy. For the bacteria, several methods are considered as the criteria to distinguish between the closet species of bacteria explained for three methods (Mayo et al. 2010).

Firstly, the phenotypic information is the fundamental information to be easy to differentiate between the closet similarity strain and expected strain with genus as the deepest level. Criteria to consider are compiled such as Gram's staining, shape, arrangement, biochemical characteristics, etc. However, the drawback of this information sometimes cannot explain the differentiation in the species level. For LAB, Gram's positive result, acid production, and catalase test are typically used to preliminary criteria to characterise this bacterial group out from the neighbour groups.

Secondly, the chemo-taxonomic marker is used based on the differentiation of some chemical substances on the cell membrane and cell wall by which cells use those chemicals for the benefit of attachment and quorum sensing.

Finally, the genotypic information is used to distinguish the maximum similarity strain which is deeper than genus level including species and strains. Various methods are used including of 16S rRNA region in only bacterial organism to identify for genus and species based on the variation of DNA base. Another region is also allowed to associate to more accuracy identification depending on the prospective bacteria. Besides, this technique is permitted for novel species identification for classification and nomenclature in advance with the criteria for the maximum similarity at 97 percent and other information to consider composed of DNA-DNA hybridization.

Probiotics can be found in various types of fermented foods; however, milk and whey are mainly substrate performing in this thesis. Then the next topic describes about the property of milk and whey in dairy products.

5. Dairy products

5.1. Milk

Milk is the white liquid produced from female mammalian species to gain the nutrients and energy to new-born babies. This liquid consists of the essential nutrients (Table 2-7) including lipid, lactose, essential amino acids, vitamins and inorganic elements to support the neonatal to survive. Moreover, maternal immunoglobulins are included as passive-naturally acquired immunity to transfer to babies, and enzyme, enzyme inhibitor, hormone, growth factor and antibacterial agents are whole received from maternal transferring. Humanity knows the way how to use the milk since about 8000 BC with both pure drinking in household and application to preserve this milk in form of fermented milk. Nowadays, over 700 million tonnes annually are consumed with 84 percent of bovine milk as the most market share worldwide. Then milk reliance is necessary as a part of the ingredient. The largest division is beverage milk for 40 percent and cheeses for 35 percent (O'Mahony and Fox 2008).

To describe milk composition, milk composes of various valuable nutrients and complex fluid containing water, protein for 1 – 20 percent, lipid for 2 – 55 percent and sugar in term of lactose for 0 – 10 percent as the major constituents. Lactose as the main sugar in milk is constructed by UDP-galactose and glucose combination in maternal metabolism with α-lactalbumin as helping protein to more reduce Michaelis constant (K_m) of glucose down a thousand folds. Likewise, other sugars remain to oligosaccharides with three to eight of monosaccharide combination such as fucose (6-deoxyhexose), N-acetylneuraminic acid, and so on. For this general information in term of physical properties is provided in the Table 2-8.

Turning to lipid component, this is essential for cell structure of the child and body's protection from cold environment with the storage in subcutaneous layer. Lipid in milk can be divided into three parts composed of neutral lipid for 98.5 percent with the member of glycerol esters, polar lipid for around 1 percent which is necessary for globule membrane and miscellaneous lipid for tiny portion by which cholesterol and lipid-soluble vitamins are included. Additionally, milk carotenoids that are grouped as the last group lipid are important for butter and cheese colouration. Fatty acids as a

part of lipid in milk usually are synthesised from the mammary gland with acetyl-CoA conjugation. The large proportion is held on hexadecenoic acid (C16:0) for 50 percent; however, unsaturated, and polyunsaturated fatty acids are found in a few contents. With the various types of lipid in milk, it can perform as emulsion dispersing in milk serum with globule diameter around 3 – 4 μm, and dairy product flavours generally contribute from fatty acids with the conversion to lactone, and keto acid.

Table 2-7. Nutrition facts of commercial milk purchased in local convenient store

Nutrition facts	**Metric**		**%Thai RDI**
1 cup (200 ml) per serving			
Energy	140	kcal	×
Energy from fat	70	kcal	×
Protein	6	g	×
Fat	8	g	12 %
Saturated fat	5	g	25 %
Cholesterol	25	mg	8 %
Carbohydrate	10	g	3 %
Total sugar	10	g	×
Dietary fibre	0	g	0 %
Sodium	85	mg	4 %
Calcium	(200)	mg	25 %
Iron	(0)	mg	0 %
Phosphorus	(160)	mg	20 %
Vitamin A	(48)	μg RE	6 %
Vitamin B1	(0)	mg	0 %
Vitamin B2	(0.26)	mg	15 %
Vitamin B12	(0.6)	μg	30 %

%Thai RDI (% Thai recommended daily intake) was the measurement that provides from the report of nutrition facts (No.182) in the year 1998 by Ministry of Health of Thailand to describe the maximum volume to intake per day. Cross sign (×) refers to no maximum limitation to intake per day from Thai requirement, and μg RE represents microgramme retinol equivalent for measurement unit. Data in parenthesis represents for data transfer from related measurement.

Table 2-8. Physical property of milk (modified from O'Mahony and Fox, 2008)

Physical property	**Value**
pH	6.5 – 6.7
Acidity	0.14 – 0.16 %
Density	1030 kg/m^3
Specific heat capacity	3880 – 4000 J/kg·K
Thermal conductivity	0.548 W/m·K
Thermal diffusivity	1.34×10^{-7} m^2/s
Surface tension	52 mN/m
Electrical conductivity	0.460 S/m
Dielectric permittivity	67.9
Dielectric loss	17.6
Refractive index	1.3440 – 1.3485
Freezing point	-0.512 to -0.550 °C
Osmolarity	0.285 osmol/kg

Casein is indispensable protein to discuss in milk as the major constituent. Casein micelles are spherical shape with size around 120 nm and mass around 10^8 Da, and 94 percent of protein and the others for low-molecular-mass components which are colloidal calcium phosphate contain in one micelle. Casein components are composed of 38, 10, 35 and 12 percent for α_{s1}-, α_{s2}-, β- and κ-casein, respectively, in bovine milk. Disulphide bond needs for casein formation causing for micelle form that α_{s1}- and β-casein lacks for cysteine and cysteine, while the others contain disulphide bonding. Besides, hydrophobicity of each casein component is essential for solubilisation in liquid whey that β-casein is the most hydrophilic peptide.

Vitamins are the trace factors containing in milk. Mostly, those vitamins are found in binding-protein form. Retinol (vitamin A) combines with β-lactoglobulin is an example. Riboflavin (vitamin B2), biotin (vitamin B7), folic acid (vitamin B9) and cobalamin (vitamin B12) are also the vitamin discovering in protein adhesion. This binding is related for intestinal-absorbing improvement. Besides, antioxidative

vitamins are also contained in milk including α-tocopherol (vitamin E) and ascorbic acid (vitamin C) to cure with free radical oxygen.

The largest part of milk is water that necessitates for colloid forming and stability. Likewise, Maillard browning, lipid oxidation, microbial growth and enzyme working uses the water as well.

With high water content, neutral pH and greater for chemical components, milk is satisfied amongst microbial growth. Thus, microbes discovered in milk can be divided into three sections including pathogenic, spoilage and beneficial microbes. From this point, milk could be used for added value with safe microbial fermentation to be certain for taste, texture, aroma, and beneficial supplementation for human's health.

5.2. Whey

Whey is the by-product of casein precipitation. One portion of milk can be separated to casein for cheese making around 10 – 20 percent, while liquid whey is 80 – 90 percent. After membrane technology, whey is the useful product to be used in various industrial propulsions. As the liquid whey, microfiltration is used for making the desalted whey and removing the fat casein out. Ultrafiltration is used for making whey protein concentrate to making commercial whey protein. Moreover, reverse osmosis is used for removing the water out from the liquid whey to more concentrated whey. Many by-products are not extinct including lactose and small fragment of protein. They are used in chemical industry as well as fuels (Cheryan 2000). Whey preparation can be separated into two categories including sweet whey and acid whey. Sweet whey is received by the addition of enzyme into milk for casein precipitation such as normal cheese production in cheese industry, while acid whey is obtained from acid addition which is agglutinated at casein isoelectric point around pH 4.6. The latter whey is found in cottage cheese production. In the USA, whey production is belonged to 94 percent of sweet whey and 6 percent of acid whey. To consider the nutrient composition of whey, around 94 percent of whey consists of water, while amount of lactose, protein, and minerals accounts for 4.5, 0.8 and 0.7 percent, respectively. They are several reports on protein composition in the whey including 48 percent for β-

lactoglobulin which is the allergenic agent for human's infant (O'Mahony and Fox 2008), 19 percent for α-lactalbumin, 20 percent for proteose-peptone, 6 percent for serum albumin and 8 percent for immunoglobulins (Kilara and Vaghela 2018).

Moreover, with some insight properties of whey protein, it can induce the hypocholesterolemic activity to protect the cholesterol absorption from non-probiotic activation. Four confirming peptides from β-lactoglobulin in cow milk including Ile-Ile-Ala-Glu-Lys, Ala-Leu-Pro-Met-His, Gly-Leu-Asp-Ile-Gln-Lys and Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys are related with this property (Nagaoka et al. 2001; Ohlsson 2010). This reaction is related by the inhibition of cell receptor at intestinal part, especially Niemann-Pick C1-like 1 (NPC1L1) as a protein carrier to uptake cholesterol to inside of enterocyte cells before sending to systematic circulation. This results in cholesterol prohibition and, subsequently, excretion out with faeces (Li and Chiang 2009). Besides, sphingomyelin molecules can reduce cholesterol uptake to blood circulation with significant exposed result performing *in vivo* rat. Additionally, the reduction of saturated fatty acid can repress plasma cholesterol. The consumption of low-fat dairy product is an example to prolong this repressing (Ohlsson 2010).

Nowadays, with the progressive technology, liquid whey from the worthless by-product in the past can be a part of today's industry. Chemicals and some food supplementations are examples. Moreover, β-lactoglobulin also results on hypocholesterolemic activity from non-bacterial origin. This by-product might be the alternative way for functional foods in advance.

Milk and whey are one of the raw materials to be produced in fermented dairy products. Therefore, general information of these diets that are expected as isolation sources and expected products are given in next heading.

6. Fermented foods

6.1. Fermented meat

Fermented foods are one of the sources of edible bacteria community, particularly LAB. Then some extraordinary metabolisms may be found from LAB fermentation. Fermented foods are classified for many groups including meat, fish, cereals, vegetables and fruit, bean, and dairy products. Then these topics are explained to LAB found in fermented foods to support for the topic of this research on cholesterol-lowering and GABA-producing activity.

Fermented meat products that use various meats such as pork, beef, and chicken as the main ingredient make these fermented meat products containing cholesterol from those meats. It is possible that in fermentation process, LAB acting the key role for fermentation might have activity to reduce cholesterol. For example, *Lactobacillus plantarum, L. pentosus, L. sake, Pediococcus pentosaceus* were observed in Nham, traditional Thai fermented pork (Kantachote et al. 2016; Visessanguan et al. 2004). Moreover, potent strains of LAB including *P. pentosaceus* HN8 and *L. namurensis* NH2 are GABA producers and cholesterol-lowering strains used as starters for producing Nham to reduce cholesterol from 130.41 ± 11.22 to 96.15 ± 11.41 mg/100 g of Nham. Both strains are probiotic candidates to inhibit many species of foodborne pathogens and to assimilate the cholesterol (Ratanaburee et al. 2013c; b). Likewise, Salami is a smoked-fermented sausage of Italy found various species of LAB including *L. sakei, L. curvatus* and *L. plantarum* (Pisacane et al. 2015). It can be concluded that some strains of LAB in fermented meats have the potential for assimilating the cholesterol, and this strongly supports for cholesterol-lowering LAB.

6.2. Fermented fish

Fish either from sea or freshwater also has cholesterol. It is predictable that fermented fish can be a source of LAB with the activity for cholesterol reduction. For instance, Budu is one of fermented sea fish originated in the southern region of Thailand. Fermented process takes a long period for several months up to 12 months and can be found different microbes depending on fermentation time. Archaea and bacteria with proteolytic and lipolytic functions are dominant microbes in Budu

fermentation. However, *Lactobacillus* spp. and *Pediococcus* spp. are found to damage for some pathogenic bacteria and also promote the taste and aroma (Laochareonsuk et al. 1990). On the other hand, fermented freshwater fish (Pla-ra) is typically found in the north-eastern region of Thailand. Various bacteria including LAB are observed such as *L. farciminis, L. salivarius* subsp. *salivarius, L. animalis, Staphylococcus* spp., *Bacillus* spp., *Micrococcus* spp. and *Tetragenococcus halophilus*. Pla-jom is one of fermented fish that similar to Pla-ra found LAB including *L. farciminis, L. salivarius* subsp. *salivarius, L. animalis, L. pentosus* and *L. plantarum* (Tanasupawat and Komagata 1995). Pla-som as the fermented whole fish is also found LAB i.e. composed of *S. salivarius, P. cerevisiae, L. plantarum* and *Enterococcus faecalis* (Hwanhlem et al. 2011). In addition, Kung-jom is a fermented shrimp that found LAB including *L. farciminis* and *L. plantarum* (Tanasupawat and Komagata 1995).

6.3. Fermented dairy products

There have varieties of fermented dairy products, while various types of milk are used such as cow, cattle, mare, and buffalo. Most fermented dairy products are lactic acid fermentation. This leads to the reduction of pathogenic bacterial contamination and generate curd to improve the different textures, and better aroma and taste. However, the aroma and taste specificity are depended on LAB strains. In addition to LAB, starters are commonly used for producing fermented milk products. Hence, *Lactobacillus* sp., *Lactococcus* sp. and *Leuconostoc* sp. are normally found in the fermented milk products. In the today's world, 20 percent of fermented products are held on fermented dairy products, particularly yoghurt is an example. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are the starters for yoghurt production. Furthermore, cheese is a product from casein precipitation that is popular source of LAB. Another example is kefir that is fermented by LAB and yeasts by putting a kefir grains in bovine milk, and diacetyl is produced for better taste. Nowadays, probiotic bacteria are popularly used for several fermented foods, especially fermented milk. For example, *Bifidobacterium bifidum* is used for commercial probiotic fermented milk.

Many candidate LAB strains are used as starters in various fermented dairy products. For instance, two main strains of LAB including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are used in yoghurt production. *S. thermophilus* uses lactose as main sugar in milk for cell energy and carbon source. Then only L-form of lactic acid is excreted to give results for aroma and taste. Meanwhile, *L. bulgaricus* is also obligate homofermentation to produce only lactic acid. Moreover, the growth of *L. bulgaricus* is activated by formic acid, folic acid and carbon dioxide production from the counterpart strain that both formic and folic acid are used for purine synthesis, and carbon dioxide is used for aspartate, glutamate, arginine and nucleotide production, while the growth of *S. thermophilus* is stimulated by the counterpart that releases free peptides. Thus, both LAB stains have the relationship in term of proto-co-operation that helps each other to survive (Bovornreungroj 2018).

In the milk industry, starters are necessary for dairy fermentation. Freeze-dry starter in nowadays is popular to be used owning to overwhelming fermentation in limited time with a good quality. Furthermore, the prediction of metabolism is indispensable for quality guarantee.

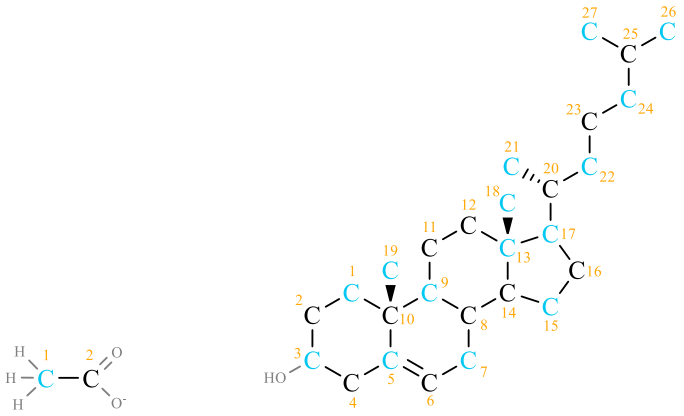
Various compounds are generated by LAB fermentation; however, some compounds should be lowering like cholesterol from raw materials. Then the remaining topics are provided to know the role of LAB for the possibility to improve fermented foods including fermented milk and whey products to be functional foods and beverages.

7. Cholesterol

Cholesterol is a biochemical compound found in mammal metabolic processes relating with cell membrane components that categorise as one type of lipids. Moreover, sterol-based hormones are derivatised from cholesterol molecule including bile salts. Humans can establish this molecule themselves in hepatocyte anabolised from lipid micromolecules. Thus, humans are not essential for receiving this outsource molecule to their bodies. Besides, the cholesterol level in blood has a relationship with CVD from atherosclerosis. Therefore, cholesterol-fortified diets should be considered before consumption (Nelson and Cox 2008).

7.1. Structure of cholesterol

Twenty-seven carbon atoms are manufactured to both steroid core and side chain to synthesise for one cholesterol molecule. Acetate consists of two carbon atoms as the substrate for synthesis. One publication was reported to extricate how to establish the cholesterol molecule is reported. To describe, the experiment was separated for two independent experiments including, the first, ^{14}C-labelled radioactive carbon atom in methyl group of acetate and another part, ^{14}C-labelled radioactive carbon atom in carboxylic group. Subsequently, those labelled acetate molecules separately put in the experimental animals were separately monitored with the basis of natural cholesterol conformation. After finishing the experiment, both data of the independent labelled-carbon atoms were synchronised to construct one cholesterol molecule and display the source of those carbon atoms, representing with blackish colour for carbon atoms from carboxylic groups, while the bluish from methyl groups (Figure 2-4) (Nelson and Cox 2008).



Acetate Cholesterol

Figure 2-4. Structure of cholesterol synthesis from acetate indicating by blackish- and bluish-labelled carbon represented to carboxylic groups and methyl groups, respectively (modified from Nelson and Cox, 2008)

7.2. Synthesis of cholesterol molecule

Sterols are one type of lipids that are essential for cell metabolism amongst the kingdom of animalia, fungi and plantae. However, only cholesterol is constructed by animal cell, while fungi and plant cell construct ergosterol and stigmasterol (Nelson and Cox 2008). For the synthesis pathway, four phases are combined for this process as shown in the Figure 2-5.

7.3. Degradation of cholesterol by microorganisms

Cholesterol can be degraded down to the fundamental micro-molecules with various reports by microorganism degradation activity. For example, *Pseudomonas* sp. NCIB 10590 can destroy this molecule down in the aerobic condition (Owen et al. 1983). Likewise, *Mycobacterium tuberculosis* is reported to have cholesterol-degrading activity to be final for water and carbon dioxide portrayed the degradation pathway in the Figure 2-6 (Ouellet et al. 2011).

Additionally, transformation of cholesterol to 4-cholesten-3-one by cholesterol oxidase as an extracellular enzyme, which is composed of either cell membrane enzyme or cytosolic enzyme (Kim et al. 2002; Saranya et al. 2014). Recently, *Lactobacillus helveticus* CD6 was found to have the activity for the degradation of cholesterol, and it was better than other recombinant LAB strains such as *L. plantarum* and *L. casei*. Cholesterol oxidase of this *L. helveticus* CD6 was detected by the reaction of hydrogen peroxide from cholesterol degradation, and intermediate substances including AD and ADD were determined from the degradation activity. This enzyme located at inside the cell as an intracellular enzyme with the specific enzyme activity at 68 U/mg, and 1 mg/ml cholesterol was completely degraded within 42 h (Ahire et al. 2012, 2013).

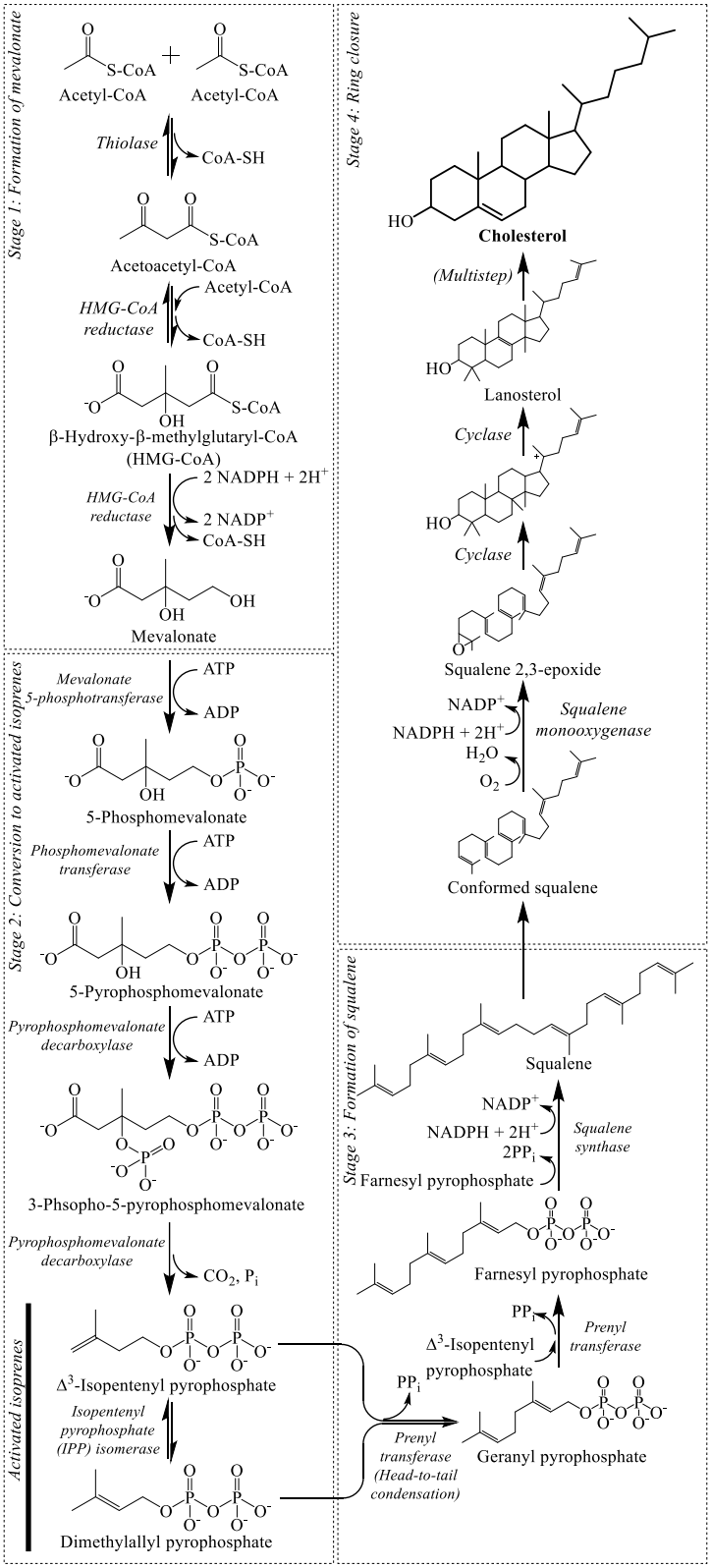


Figure 2-5. Four steps of cholesterol synthesis from the acetate monomer including formation of mevalonate, conversion to activated isoprenes, formation of squalene and ring closure, respectively (modified from Nelson and Cox, 2008)

Cholesterol oxidase can be categorised for two groups depending on the active sites including non-covalent-bound flavin adenine dinucleotide (FAD) form as type 1 and covalent-bound FAD form as type 2 (Figure 2-7). Type 1 enzyme is classified as glucose-methanol-choline (GMC) found in *Streptomyces* sp. and *Brevibacterium sterolicum*, while type 2 is found in *Brevibacterium* spp. Both enzymes require FAD to impulse the reaction from cholesterol as a substrate to 5-cholesten-3-one before terminating to 4-cholesten-3-one with hydrogen peroxide as by-product. The active site of type 1 enzyme is propelled with the reaction of imidazole to hydroxyl group of cholesterol by hydrogen bond reaction of His447 for hydrogen donation of imidazole to hydroxyl group, while type 2 enzyme is impelled by Arg477 with a similar reaction. However, the midpoint reduction potential of both enzymes is also different with -278 mV in type 1 and -101 mV in type 2. Another factor to drive the reaction relies on oxygen assessment that both enzymes are designed themselves with oxygen tunnel both open and closed positions to limit the oxygen amounts (Sampson and Vrielink 2003).

Numerous publications have reported for cholesterol assimilation by microorganisms. For example, some strains of yeasts can utilise cholesterol in synthetic medium fortified with cholesterol including *Saccharomyces cerevisiae* 832, *S. cerevisiae* KK1 and *Isaatchenkia orientalis* KK5.Y.1, they were candidate probiotics and assimilated cholesterol in the medium to be empty (Psomas et al. 2003). Besides, cholesterol assimilation by LAB is indispensable characteristics for probiotic candidates. For instance, *Lactobacillus fermentum* KC5b assimilated 14.8 mg of cholesterol per 1 g of cell dry weight in MRS fortified with 100 mg/L cholesterol and 4 g/L oxgall at 37 °C for 12 h of cultivation (Pereira and Gibson 2002). *L. plantarum* NCIMB702656 assimilated 38.99 ± 4.87 percent of cholesterol from the supplemented medium under incubating condition at 37 °C for 24 h (Tomaro-Duchesneau et al. 2014). While *L. plantarum* B0007 assimilated 167.03 ± 8.20 mg/L of cholesterol in supplemented medium containing MRS medium containing 300 mg/L cholesterol and 3 g/L oxgall at 37 °C for 20 h of incubation (Wang et al. 2014).

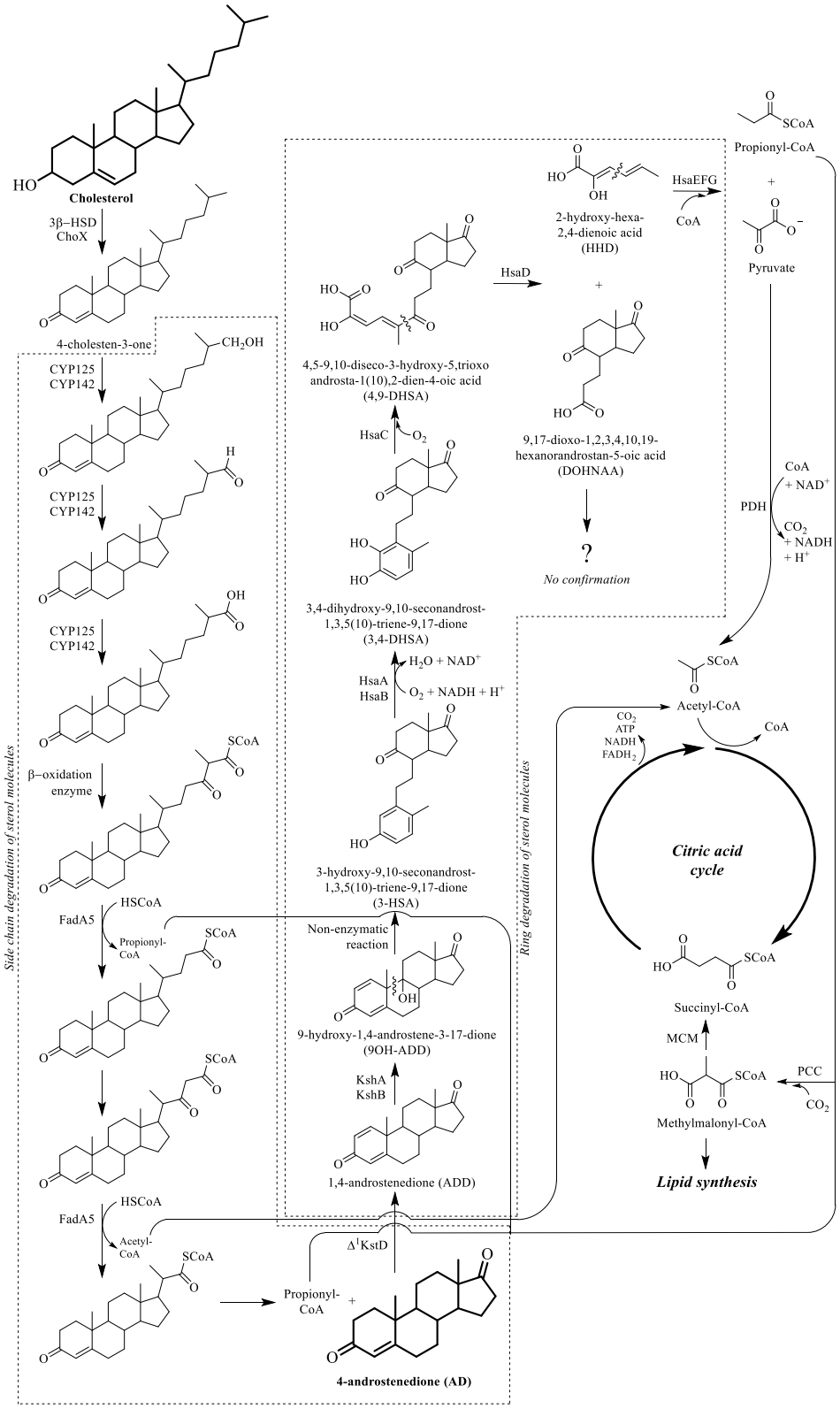
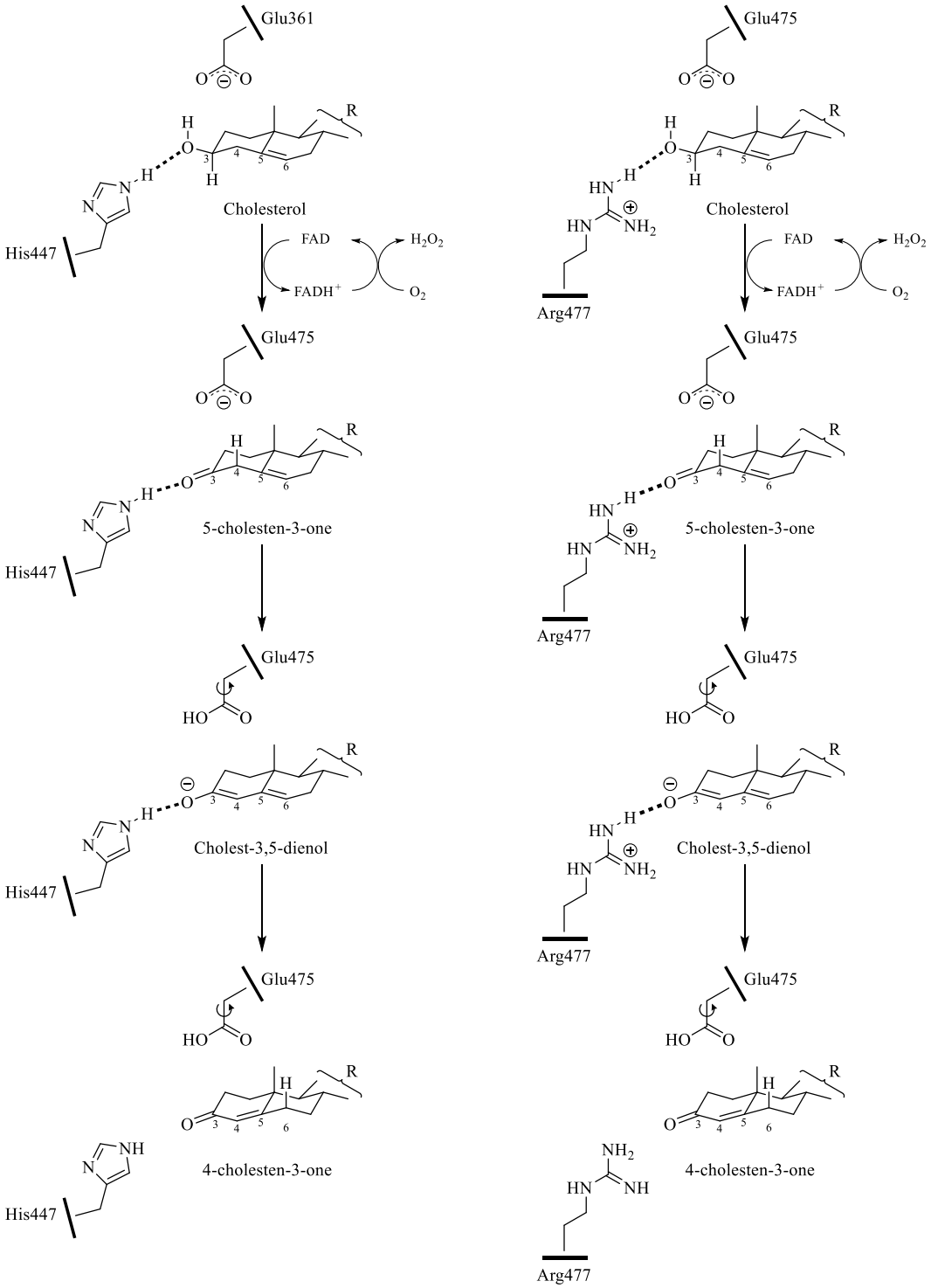


Figure 2-6. Cholesterol degradation pathway of both side chain and ring nucleus degradation subscribed from *Mycobacterium tuberculosis* (modified from Ouellet et al., 2011)



Type 1:
non-covalent-bound FAD-dependent cholesterol oxidase

Type 2:
covalent-bound FAD-dependent cholesterol oxidase

Figure 2-7. Cholesterol oxidase reaction of both types including type 1 as non-covalent-bound and type 2 as covalent-bound FAD enzyme (modified from Sampson and Vrielink, 2003)

By the way, some publications declare on cholesterol-degrading bacteria with similar mechanisms as above. For example, *Agrobacterium* spp. M4 utilised cholesterol in the medium for 9 days to be empty and found important metabolite namely 4-cholesten-3-one (Yazdi et al. 2001). While the degradation activity in *Bacillus subtilis* SFF34 isolated from Korean traditional fermented fish degraded cholesterol for 60 h to be empty by cholesterol oxidase, and important metabolite was found as 4-cholesten-3-one (Kim et al. 2002).

8. Bile salts

Bile salts are the derivatised molecules from generating of cholesterol by body metabolisms, especially hepatocyte. However, constructed bile salts by human metabolisms can be deconjugated by some bacteria, particularly some probiotic LAB strains that have bile salt hydrolase (BSH). This topic describes on bile salt synthesis and deconjugation.

8.1. Synthesis of bile salts

Primary bile salts including glycocholic acid (GCA) and taurocholic acid (TCA) are synthesised from cholesterol in hepatic part and collected those compounds in gall bladder to secret to duodenum, which is the initial part of intestine, to bind with some lipid molecules. In the intestinal part, some bacteria change primary from to secondary bile salts which are included glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA). Bile salts are then re-absorbed in the ileum part of intestine to circulate for re-using these molecules in the next opportunity (Hofmann and Mysels 1992). Dominant bile salts that are normally used to study their effects on probiotic examination include GCA, GDCA, TCA and TDCA. For synthesis mechanism in hepatic cells is displayed in the Figure 2-8.

8.2. Deconjugation of bile salts

Bile salt hydrolase (BSH) is one of the various enzymes found in probiotic contestant strains, especially LAB to have for avoiding the severe situation from bile salt damaging to their cell membrane by exposition of BSH construction to break down those molecules. Outgrowth from this situation is to protect the absorption of lipid to lymphatic system before going to circulatory system (Erkkilä and Petäjä 2000; Pennacchia et al. 2004). Activity of breaking down enzyme for bile salt hydrolase is depredation between amide bond conjugation as shown in the Figure 2-9.

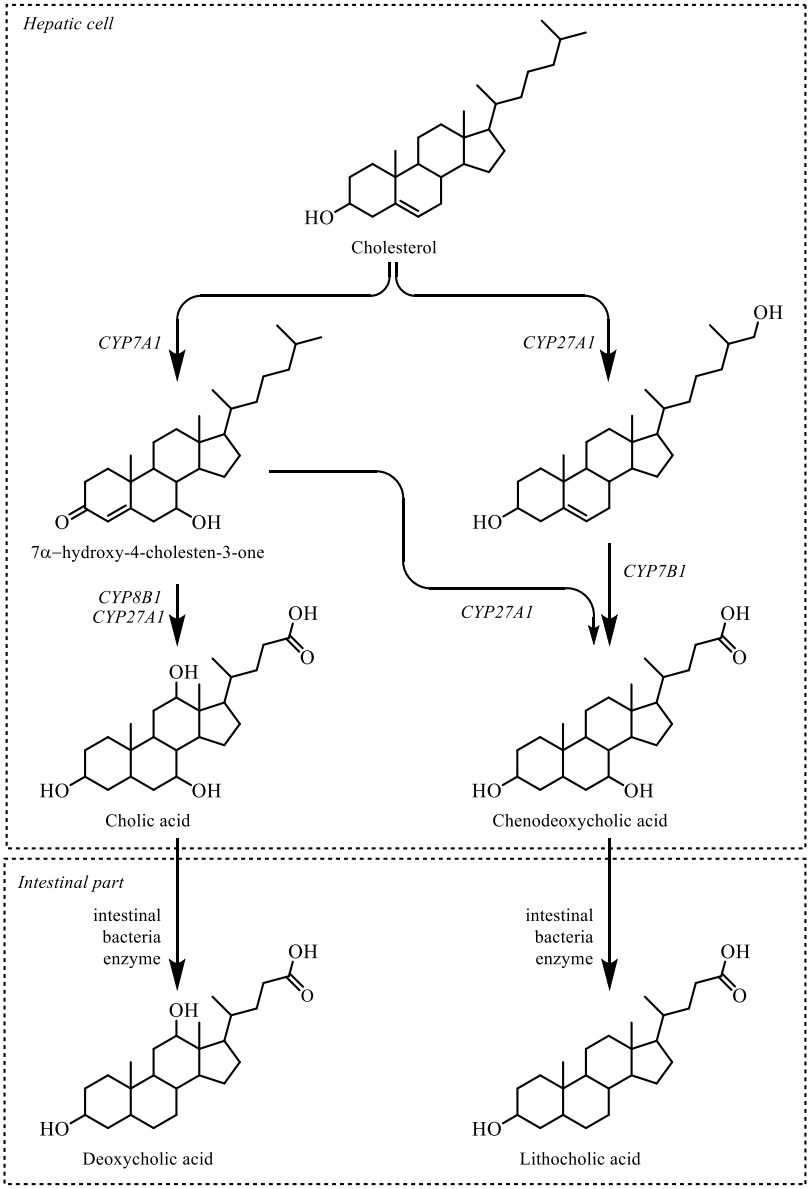


Figure 2-8. Bile salt synthesis from cholesterol in hepatic organ (modified from Li and Chiang, 2009)

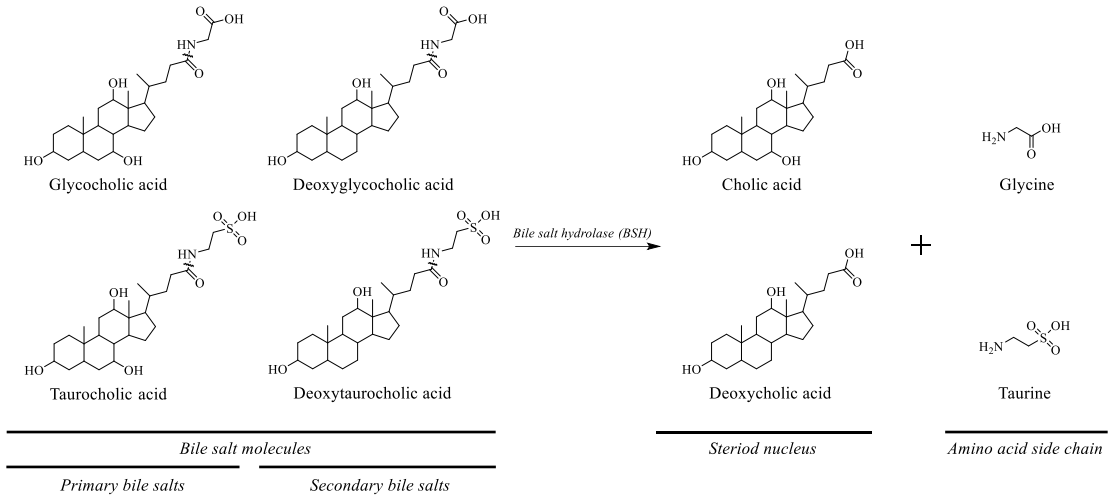


Figure 2-9. Deconjugation of bile salt hydrolase (BSH) activity on four important bile salt molecules including glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) (modified from Bhange et al., 2014)

9. γ-Aminobutyric acid (GABA)

GABA is non-protein amino acid molecule. After uptake this compound to human's body, it acts as major inhibitory neurotransmitter related with nervous system. This compound processing is acted by secretion from pre-synaptic nerve to bind with the receptor on post-synaptic membrane to activate influx of chloride ion and efflux of potassium ion. This flow reflects to nervous potent in term of hyperpolarisation which is related with membrane stimulus to reach the threshold action of nervous domination. From this viewpoint, the increase of GABA content in pre-synapsis can enhance the efficiency of interneuron communication and reduce the delayed time in the synaptic cleft (Pack 2007; Wong et al. 2003). Moreover, antidepressant, hypotension, and diuretic symptoms also display. This substance is mainly established from the irrevocable conversion of glutamic acid by intracellular glutamic acid decarboxylase (EC 4.1.1.15; Figure 2-10) requiring pyridoxal-5'-phosphate as a coenzyme. Various organisms contain this enzyme to change the excess glutamic acid to GABA such as bacteria, yeasts, fungi, and humans.

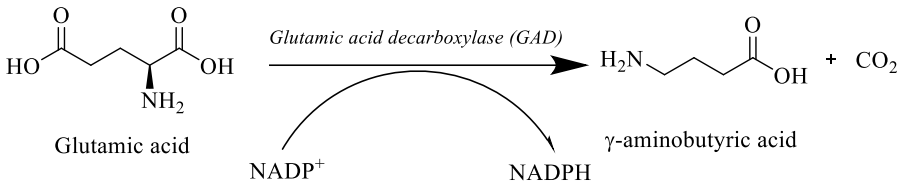


Figure 2-10. GABA production from glutamic acid by glutamic acid decarboxylase

This substance, GABA, has the potential for pharmaceutical and diet aspects. Nowadays, it is found several functional foods fortified with GABA as the supplement. For example, tea, fermented grape, Thai fermented pork sausage, sourdough, cheeses, Kimchi, black raspberry juice and rice bran are found GABA as the supplement to improve to be functional foods. Most of functional foods are supplied GABA by LAB fermentation using starter(s). For instance, *L. brevis*, *L. lactis*, *L. paracasei*, *L. buchneri*, *L. helveticus*, *L. namurensis* and *P. pentosaceus* are examples for GABA-producing LAB (Hebert et al. 2015).

10. Angiotensin-converting enzyme (ACE) inhibitor

Angiotensin-converting enzyme (ACE) is the zinc-metalloenzyme with the activity of peptidyldipeptide hydrolase (EC 3.4.15.1) in the group of renin-angiotensin system to control between vasodilatation and vasoconstriction. Especially the mechanism of ACE (Figure 2-11), this enzyme relates to blood pressure regulation. Normal mechanism of this system is briefly described. Renin enzyme works by cleaving the angiotensinogen with 452 amino acid length, but the first 12 amino acids of C-terminal side is important side to cut at the position 10th from Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-440 amino acid sequence to Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu as decapeptide and long residue. This enzyme is produced from juxtaglomerular cell of kidney. This decapeptide namely angiotensin-1 is cleaved by ACE to octapeptide sequencing Asp-Arg-Val-Tyr-Ile-His-Pro-Phe as angiotensin-2 and His-Leu. The angiotensin-2 induces blood pressure to hypertension and releases aldosterone secretion from adrenal gland cortex to efflux the sodium out from the urinary tract back to the vessel. Moreover, with the supporting property to increase vasoconstriction, ACE also cleaves bradykinin which is the enzyme to activate the reduction of blood pressure by the vasodilatation (Mozzi et al. 2010).

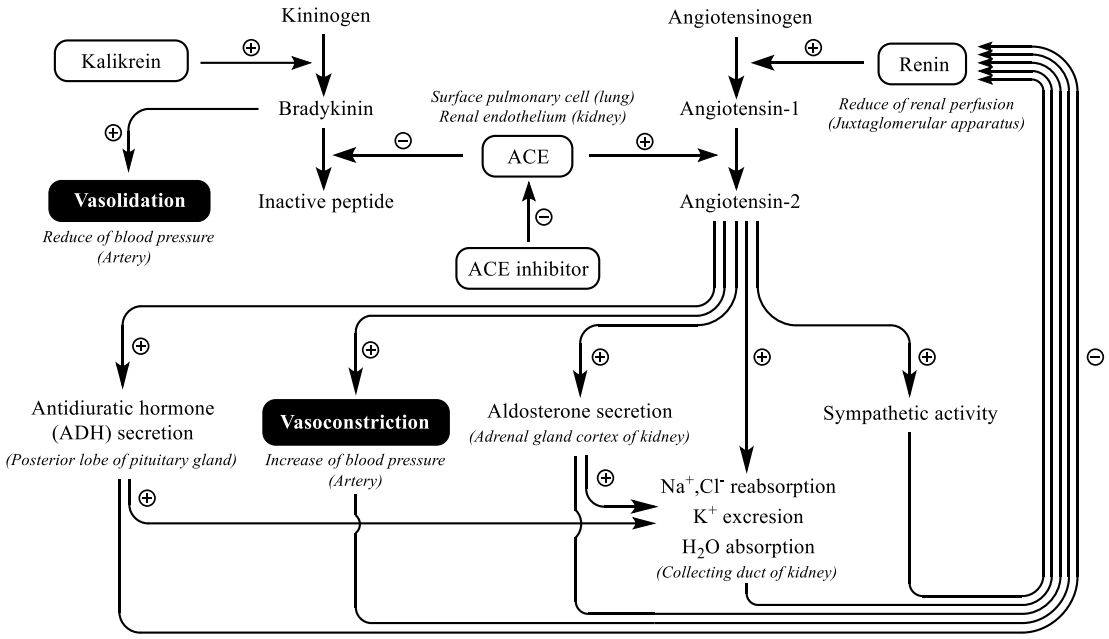


Figure 2-11. Renin-angiotensin-bradykinin mechanism (modified from Mozzi et al., 2010; de Almeida and Coimbra, 2019)

For the medication nowadays to antihypertension, drug namely Captopril and Lisinopril mimicked from amino acid structure are cleaved to proline and (2S)-3-acetylthio-2-methylpropanoyl chloride to obstruct the ACE working. Besides, several publications have reported for peptides from casein breakdown to have a relationship with antihypertension. Peptide lists including Ile-Pro-Pro, Val-Pro-Pro, Leu-Pro, Val-Pro, Ala-Pro, Pro-Pro, Val-Leu and Tyr-Pro are the functional peptides to control ACE by binding to active site to repress the activity of vasoconstriction (Hagi et al. 2016). Moreover, to insight consideration, some amino acids compose to those peptides are related with inhibitory effect including aromatic amino acids composed of tryptophan, phenylalanine, tyrosine and proline and positive charge amino acids composed of arginine and lysine. Those peptides are found from casein breakdown by the proteolysis of bacteria, especially LAB. For example, *L. helveticus* CP790 produced casokinin tripeptide or lactokinin as ACE inhibitor from casein degradation in form of Ile-Pro-Pro and Val-Pro-Pro, and IC_{50} was around 100 – 500 µmol/L. These tripeptides also showed the result to directly reduce systolic and diastolic blood pressure. To be concluded, the functional peptide is one of the alternative ways to reduce hypertension which is sufficient and convenient to find (Mozzi et al. 2010).

11. Antioxidative compounds

Antioxidative molecules are one of various qualifications that microbes can generate to fight with free radical molecules to cure themselves to survive from many free radical molecules. To know the potential of antioxidative agents, several detections are created including with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobiz (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and oxygen radical antioxidant capacity (ORAC). By the way, the number of phenolic compounds is detected also with the method namely total phenolic content (TPC).

DPPH which is the ready-to-use radical compounds behaves in stably purplish colour. When the reaction is activated by some antioxidative agents, these compounds are going to reduce this radical form by taking proton molecule from antioxidants on the free radical position causing for colour reduction to light yellowish tone (Figure 2-12) (De Oliveira et al. 2014). Turning to ABTS, this method is familiar with DPPH, but different point is related with the generation of ABTS radicals. To describe, stable form of ABTS in green-tea colour is activated by potassium peroxodisulfate ($K_2S_2O_8$) at least 16 hours to be activated form of ABTS radicals in greenish blue colour (Figure 2-13A). When the radical form of ABTS is reacted with an antioxidative agents, the reaction is turned from greenish blue to colourless (Figure 2-13B). Moving to ORAC (Figure 2-14), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) substance is used for immediately generated to free radical oxygen molecules at 37 °C. Concept of the determination is related with AAPH molecule associated by quenching the fluorescein molecule to reduce the light emission. Nevertheless, the radical molecules are prohibited by antioxidant molecules. Then this quenching by these radicals is not happened. The light emission is still revealed, and the activity can be detected.

The quantities of phenolic compounds are detected by TPC method. Gallic acid is the popular compound to use for reporting as gallic acid equivalents. Folin-Ciocalteu reagents is the important reagent to detect phenolic compounds using phosphomolybdate and phosphotungstate with the changing reaction (Figure 2-15) (Agbor et al. 2014).

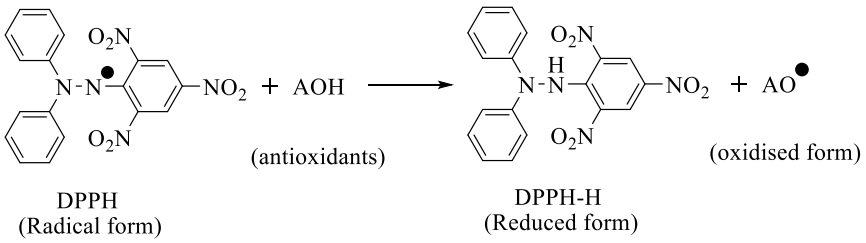


Figure 2-12. Chemical reaction of DPPH method by antioxidants (modified from De Oliveira et al., 2014)

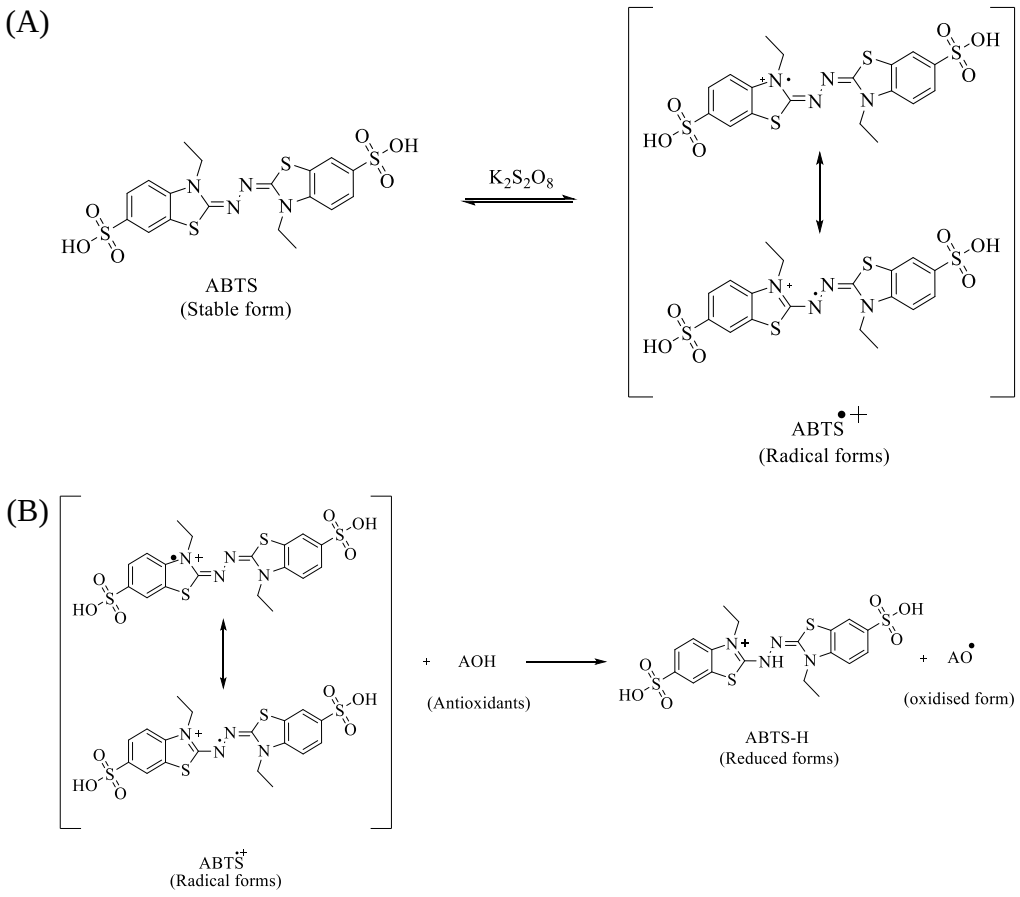


Figure 2-13. (A) Generation of ABTS stable form by $K_2S_2O_8$ to $ABTS^{\cdot+}$ radical form, and (B) antioxidant reaction of ABTS radical form by antioxidants (modified from De Oliveira et al., 2014)

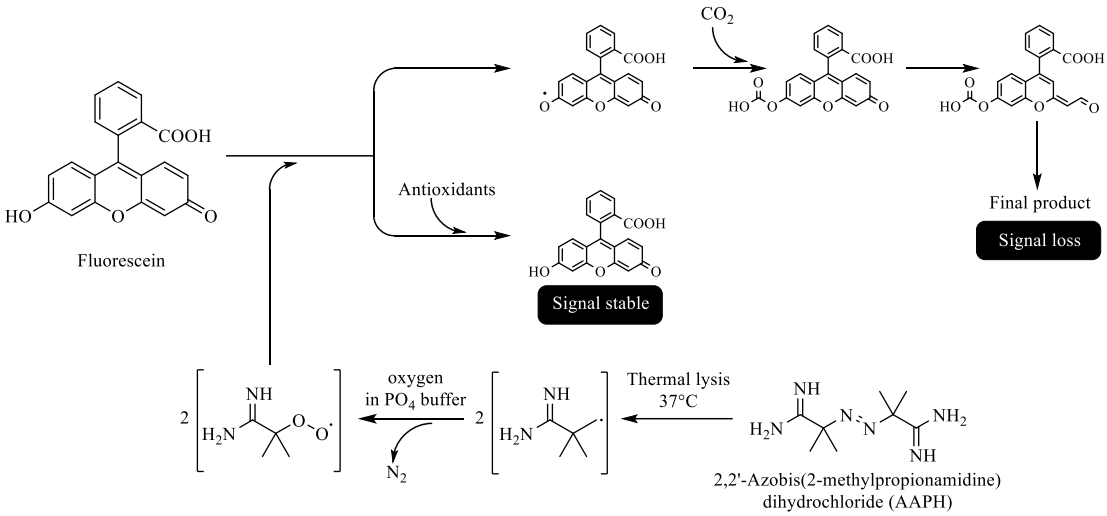


Figure 2-14. Antioxidant activity in ORAC reaction (modified from Glazer, 1990; Ou et al., 2001)

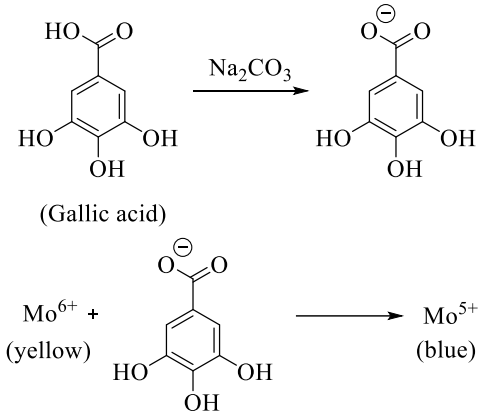


Figure 2-15. TPC reaction by gallic acid as authentic antioxidant with colour formation from antioxidant (modified from Agbor et al., 2014)

CHAPTER 3

RESEARCH METHODOLOGY

1. Directory of materials and equipment used

1.1. Materials

1. Beakers (50, 100, 250, 500 and 1000 ml, Pyrex)
2. Conical tubes (15 and 50 ml, Eppendorf)
3. Cylinders (100, 500 and 1000 ml, Pyrex)
4. Duran bottles (100, 250, 500, 1000 ml, Pyrex)
5. Erlenmeyer flasks (125, 250, 500, 1000 ml, Pyrex)
6. Glass petri dishes (50×17 and 100×20 mm, Pyrex)
7. Glass vial (2 ml, Agilent, USA)
8. HPLC columns (Hypersil ODS C_{18}, 79926OD-584, 250×4.0 mm, 5 µm; Agilent, USA; Nucleosil 100-5 C_{18} reversed phase, 720014.40, 250×4.0 mm, 5 µm; Macherey-Nagel, Germany)
9. Iron loop
10. Microtiter plate (96 wells, Nunc)
11. Microtiter plate black well (96 wells, Greiner Bio-one, Germany)
12. Microtubes (300, 1500, 5000 µl, Eppendorf and Nest, China)
13. Plastic tips for autopipette (10, 200, 1000 and 5000 µl, Nest, China; Eppendorf)
14. Screw cap test tubes (10×75, 16×150 and 25×200 mm, Pyrex)
15. Silica gel 60 F_{254} (Merck, Germany)
16. Test tubes (10×75 and 16×150 mm, Pyrex)
17. Volumetric flasks (25, 50, 100, 200, 500, 1000, 2000 ml, Pyrex)
18. Whatman No.1 filter paper (Whatman, UK)

1.2. Equipment

1. Absorbent microplate reader (LUMIstar Omega, BMG Labtech, Germany)
2. Autoclave (SX-700, Tomy, Japan)
3. Autopipettes (0.1–10, 2–20, 20–200, 100–1000 and 1000–5000 µl, Eppendorf)
4. Balances (Denver, USA; XR 205SM-DR, Precisa, Switzerland)
5. Bench top centrifuge (Thermo Scientific, USA)
6. Biosafety cabinet class II (Astec Microflow, UK)
7. Block heater (Stuart, UK)
8. Centrifuge evaporator (Centrivap, Labconco, USA)
9. Ultra-low temperature freezer (New Brunswick scientific, USA)
10. Horizonal gel electrophoresis (Select BioProducts, Taiwan)
11. Fluorometric microplate reader (Colona Electric SH-9000 series, Japan; Enspine, Perkin Elmer, USA)
12. High-performance liquid chromatography with fluorescence detector (1200 series, Agilent, USA)
13. High-performance liquid chromatography with diode array detector (1200 series, Agilent, USA)
14. Hot air oven (Binder, Germany)
15. Incubator (Gallenkamp, UK)
16. Light microscope (CX-21, Olympus, Japan)
17. Microfuge (Corning, Korea)
18. Multichannel autopipette (20 – 200 µl, Corning)
19. pH meter (DOCU-pH, Sartorius, Germany)
20. Portable pH meter (LAQUAtwin, Horiba, Japan)
21. Refrigerated centrifuge (5804R, Eppendorf)
22. Spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA)
23. Thermal cycler (T100^{TM}, Bio-rad, USA)
24. Ultra-sonicator (Sonics, USA)
25. UV transilluminator (Vilber Lourmat, France)
26. Water bath (Julabo, Germany)
27. Stomacher (Seward, UK)
28. Vortex (Scientific Industries, USA)

2. Directory of chemicals and reagents used

2.1. Chemicals and culture media

1. (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, USA)
2. 1 kb Gene Ruler (Thermo scientific, USA)
3. 2,2'-Azinobiz(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma, USA)
4. 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, Wako, Japan)
5. 2,2-Diphenyl-2-picrylhydrazyl (DPPH, Sigma, USA)
6. 2,4,6-Trimethylpyridin (Sigma, USA)
7. 2-Mercaptoethanol (Loba Chemie, India)
8. 30% Hydrogen peroxide (H_2O_2, Merck, Germany)
9. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, USA)
10. 4-Aminoantipyrine (Sigma, USA)
11. 50x TAE buffer (Bio-rad, USA)
12. 85% Glycerol (Merck, Germany)
13. 85% Potassium hydroxide (RCI Labscan, Thailand)
14. ACE enzyme (A6778-0.1UN, 100 mU, Sigma, USA)
15. Agar
16. Agarose (1^{st} Base, Malaysia)
17. Ammonium sulphate ($(NH_4)_2SO_4$, Merck, Germany)
18. Beef extract (Difco, USA)
19. Boric acid (Merck, Germany)
20. Bovine serum albumin (Sigma, USA)
21. Bromocresol green (BDH, UK)
22. Bromocresol purple (Merck, Germany)
23. Cholesterol (Sigma, USA)
24. Cholesterol-PEG600 (Sigma, USA)
25. Commercial citric acid
26. Coomassie brilliant blue G250 (Merck, Germany)
27. Cyanocobalamin (Sigma, USA)
28. de Man Rogosa and Sharpe (MRS, Merck, Germany)
29. D-glucose (Ajax, Australia)

30. Dimethyl sulfoxide (DMSO, Merck, Germany)
31. di-Potassium hydrogen phosphate (K_2HPO_4, Merck, Germany)
32. Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$, Merck, Germany)
33. Fluorescein sodium salt (Sigma, USA)
34. Folin-Ciocalteu reagent (Merck, Germany)
35. Gallic acid (Sigma, USA)
36. Gel Green nucleic acid gel stain (Vivantis, Malaysia)
37. Glutamic acid (Sigma, USA)
38. Glyceryl tributyrate (Sigma, USA)
39. Glycocholic acid (GCA, Sigma, USA)
40. Glycodeoxycholic acid (GDCA, Sigma, USA)
41. Hippuryl-Histidyl-Leucine (Sigma, USA)
42. Horseradish peroxidase (516531-5KU, 280.0 U/mg, Merck, Germany)
43. L-ascorbic acid (Fisher Scientific, USA)
44. Lysozyme (Vivantis, Malaysia)
45. MaestroSafe Nucleic Acid loading dye (Maestrogen, Taiwan)
46. Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$, Merck, Germany)
47. Manganese sulphate monohydrate ($MnSO_4 \cdot H_2O$, Merck, Germany)
48. Monosodium glutamate (MSG, local commercial store)
49. Ninhydrin (Merck, Germany)
50. O-phthaldialdehyde (OPA, Sigma, USA)
51. Pancreatin (Sigma, USA)
52. Paraffin oil (Labchem, USA)
53. Pasteurised milk
54. Pepsin (sigma, USA)
55. Peptone from meat (Merck, Germany)
56. Peptone water (Merck, Germany)
57. Phenol (Merck, Germany)
58. Phenolphthalein ($C_{20}H_{14}O_4$, Farmitalia Carlo Erba, Italy)
59. Polysorbate 80 (Merck, Germany)
60. Potassium chloride (Merck, Germany)
61. Potassium cyanide (Merck, Germany)

62. Potassium di-hydrogen phosphate (KH_2PO_4, Merck, Germany)
63. Potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$, Kemaus, Australia)
64. Potassium hydrogen phthalate ($KHC_8H_4O_4$, Merck, Germany)
65. Potassium hydroxide (RCI Labscan, Thailand)
66. Potassium peroxodisulfate ($K_2S_2O_8$, Merck, Germany)
67. Skim milk (HiMedia, India)
68. Sodium acetate (CH_3COONa, Merck, Germany)
69. Sodium carbonate (Na_2CO_3; Ajax, Australia)
70. Sodium chloride (Merck, Germany)
71. Sodium hydroxide (Merck, Germany)
72. Soluble starch (Difco, USA)
73. Sucrose (BDH, UK)
74. Taurocholic acid (TCA, Sigma, USA)
75. Taurodeoxycholic acid (TDCA, Sigma, USA)
76. Titriplex® III (Merck, Germany)
77. Tri-ammonium citrate ($C_6H_{17}N_3O_7$, Merck, Germany)
78. Tris·HCl (Fluka, USA)
79. Triton X-100 (Merck, Germany)
80. Yeast extract granulated (Merck, Germany)
81. Zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$, Merck, Germany)
82. γ-aminobutyric acid (GABA, Sigma, USA)

2.2. Solvents and acids

1. 2-Propanol (RCI Labscan, Thailand)
2. 37% (w/w) fuming hydrochloric acid (Merck, Germany)
3. 95-97% Sulphuric acid (Merck, Germany)
4. Absolute ethanol (Merck, Germany)
5. Acetonitrile (RCI Labscan, Thailand)
6. Butan-1-ol (RCI Labscan, Thailand)
7. Deionised water
8. Glacial acetic acid (Merck, Germany)
9. Hexanes (RCI Labscan, Thailand)

10. Methanol (Merck, Germany)
11. *ortho*-Phosphoric acid (Riedel-de Haën, Germany)

2.3. Reagents

1. 3% hydrogen peroxide for catalase test reagent
2. Bradford reagent for protein determination
3. Gram's stain reagent group (Crystal violet, Gram's iodine, Safranin)
4. Phenolphthalein reagent for acid titration

2.4. Test kit sets

1. API 50 CHL (Ref 50410, BioMérieux, France)
2. DNA extraction kit (QIAamp® DNA mini kit, Qiagen, Germany)
3. Gel & PCR Purification System (Biofact, South Korea)
4. HotStar HiFidelity Polymerase kit (Qiagen, Germany)
5. Primer set (27F and 1492R; 1st Base, Malaysia)

3. Isolation and selection of lactic acid bacteria (LAB)

3.1. Isolation of LAB from fermented foods and raw milk

Lactic acid bacteria (LAB) from various fermented foods and raw milk were separated depending on state of matter which remained between solid and liquid. 25 g of solid sample was weighed and combined with 225 ml of 1 g/L of peptone water which specified for diet-inhabited microbes. This mixture was entirely smashed by stomacher (Seward, UK) at 230 rpm for 30 s. One loopful from this mixture was authorised for eight-dimensional streakings as shown in the Figure 3-1 to increase the possibility of rarely found cells on MRS agar (Topic 2, Appendix A) indicating with 0.4 mg/ml bromocresol purple. Temperature at 35 °C for 24 – 72 h was necessitated for microbial growth; meantime, liquid samples were directly assayed, streaked on the MRS agar, and incubated at familiar condition.

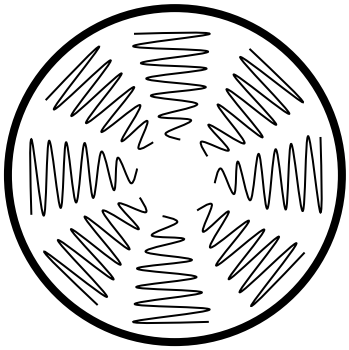


Figure 3-1. Eight-dimensional streaking model for bacterial isolation

3.2. Selection of isolated LAB

The characterisation of LAB to select out from the mixing colony types contained for three important characters including acid production, Gram's positive result, and negative catalase. To begin with acid production, MRS agar supplemented with 0.04% (w/v) bromocresol purple isolation medium (Topic 3, Appendix A) was shifted from purplish to yellowish colour around expected colonies. For Gram's positive result in the next, Gram's staining (Topic 1, Appendix B) should be illustrated purplish-shaped bacterial morphology as if Gram's positive bacteria. Finally, catalase assay performed as no gas bubble generation for the negative result (Topic 2, Appendix B).

When the suspicious bacteria were justified the satisfied results as LAB, bacterial preservation needed to prolong the life by storage in 20% (v/v) glycerol and preserved at -80 °C condition as stock culture. For working culture, viable LAB were stabbed in the tube of MRS medium and preserved at 4 °C.

To re-culture, LAB isolates were activated by streaking on MRS medium. Temperature at 35 °C for 24 – 48 h needed for bacterial growth. 10 ml of MRS broth were performed for bacterial preparation and incubated at 35 °C for 18 h.

4. Inspection of cholesterol-lowering LAB

This inspection composed of three steps from primary to tertiary inspections to find the most potential cholesterol-lowering LAB.

4.1. Primary inspection

After the preserved stocks were prepared by streaking on MRS medium, selected LAB were harvested by centrifugation at 5,000 × g for 10 min to discard the supernatant out and re-suspended in 8.5 g/L of normal saline solution (NSS) (Topic 13, Appendix A). Washing step was twice performed to eliminate the previous medium out before suspending these cells in LAB basal medium supplemented with 100 µg/ml cholesterol and 0.1% (w/v) yeast extract (Topic 5, Appendix A). 3% (v/v) inoculum of each contestant LAB was cultured in 8 ml of LAB basal medium in 8-ml-sizing screw cap tube to make microaerobic condition as Figure 3-2A. Incubation at 35 °C for 72 h was implemented before inspected each loopful culture by streaking on the MRS agar medium as Figure 3-2B. Two-third survival growth of LAB strains were the criteria for passing to the next experiment.

4.2. Secondary inspection

For the secondary screening, the procedure was familiar with the primary screening, but application medium was changed to LAB basal medium containing 100 µg/ml cholesterol without yeast extract (Topic 6, Appendix A).

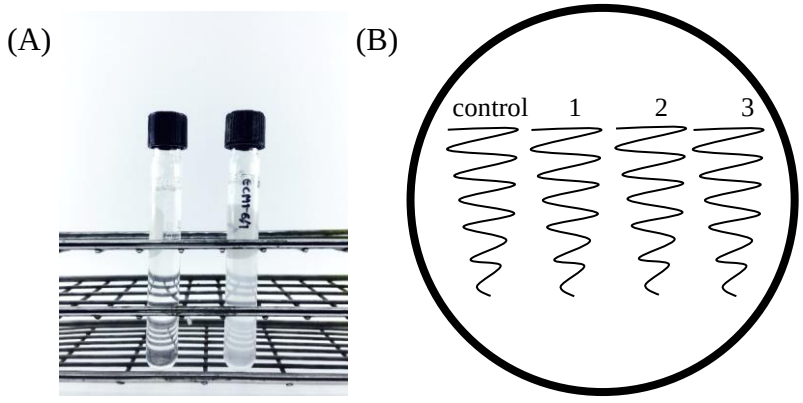


Figure 3-2. (A) LAB basal medium including (left) no inoculum and (right) inoculum, and (B) survival contestant LAB inspection on MRS agar medium

4.3. Tertiary inspection

The remaining contestant LAB were tested with this experiment. After cultivating the contestant bacteria, the bacteria were harvested by centrifugation at 5,000 × *g* for 10 min and twice washed with NSS before suspending in the medium. Media were composed of two types including LAB basal medium supplemented with 100 µg/ml cholesterol (Topic 5, Appendix A) as a carbon and energy source for treatment set and LAB basal medium (Topic 4, Appendix A) without addition of cholesterol as control set showing (Figure 3-3). 3% (v/v) inoculum of culture turbidity for OD_{660} at 1.0 was cultivated in both media. Temperature at 35 °C for 72 h was used for incubation. After incubation, the viable cells in both culture broths were counted on MRS agar. Results were compared between the control and treatment sets to observe cell proliferation in treatment.

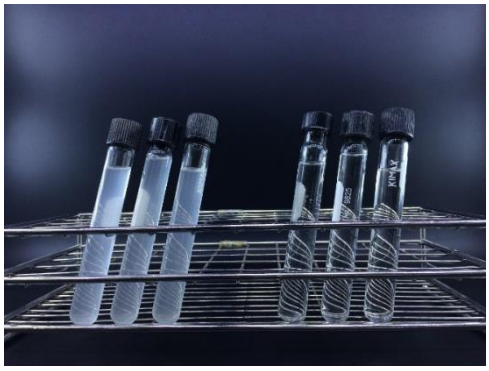


Figure 3-3. Tertiary screening medium set (left) LAB basal medium supplemented with 100 µg/ml cholesterol as treatment set, and (right) LAB basal medium as control set

5. Use of cholesterol by potent LAB

Selected LAB strains from tertiary screening were further tested. The experimental design composed as the list and in the Figure 3-4.

1. Sample set composed of contestant strains to culture in LAB basal medium supplemented with 100 µg/ml cholesterol-PEG600 (Topic 7, Appendix A). After finishing 72-h incubation, the culture was separated in two parts in the following.
   1.1. Cholesterol examination in culture supernatant (Topic 3.2, Appendix B)
   1.2. Cholesterol examination in cell pellets (Topic 3.2, Appendix B)

2. Abiotic control set composed of two parts as follows.
   2.1. LAB basal medium supplemented with 100 µg/ml cholesterol-PEG600 (Topic 7, Appendix A) represented as control for cholesterol degradation from non-inoculated culture.
   2.2. LAB basal medium (Topic 4, Appendix A) was used as control set for blank of topic 1.1 and 2.1
3. Control set was the treatment containing culture representing 0 h incubation of the whole treatment set.
4. Standard set was used for standard regression preparation to compare the quantity of cholesterol in treatment set.

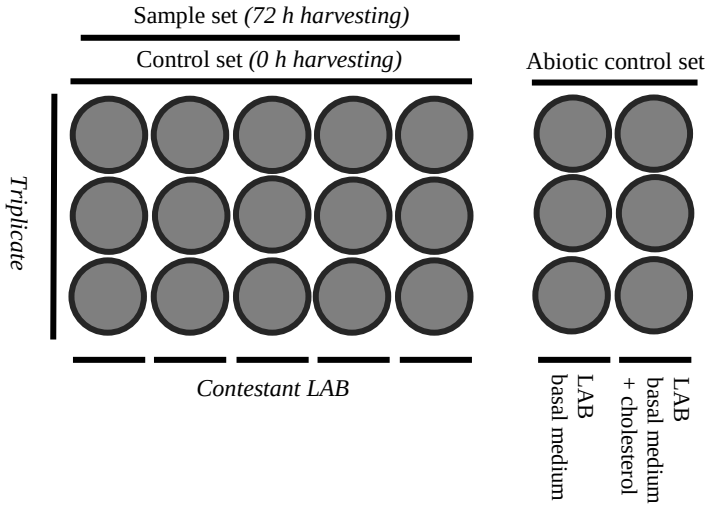


Figure 3-4. Experimental design for the use of cholesterol by potent LAB

After preparation contestant LAB in MRS broth, the bacteria were harvested by 5,000 × *g* for 10 min centrifugation and twice washed with NSS. The LAB basal medium supplemented with 100 µg/ml cholesterol (Topic 7, Appendix A) in 500 ml of one massive volume bottle before distributing to 50 ml of each bottle for equal concentration. The bacteria were finally re-suspended in the LAB basal medium supplemented with 100 µg/ml cholesterol before cultivation by 3% (v/v) inoculum of OD_{660} at 1.0 turbidity adjustment in 50 ml with familiar medium. This cell suspension was distributed to each 8-ml-sizing screw cap tube in triplicate. Moreover, the abiotic control set media were also distributed to each tube in triplicate. Condition at 35 °C of temperature for 72 h in microaerophilic condition was allowed.

Remaining culture was harvested at 0 h as control set followed (Topic 3.2, Appendix B) procedure. Briefly, the culture was harvested by 5,000 × g for 10 min centrifugation to separate cells and supernatant out. Supernatant was collected at -20 °C; meantime, cell pellets were twice washed by NSS and broken by ultra-sonication (Sonics, USA) in 1 ml of hexane suspension at 60% amplitude with 25 s and 5 s for on and off conditions, respectively. Cell debris had separated by centrifugation at 12,000 × g for 10 min at 4 °C from hexane solution before applying to cholesterol determination. Furthermore, abiotic control set both LAB basal medium with and without cholesterol were collected and stored at -20 °C for cholesterol determination as control. After finishing the incubation in sample set, 5 ml of each selected strain was harvested (Topic 3.2, Appendix B).

For the determination step followed the procedure (Topic 3.3, Appendix B). Shortly, 1 ml of sample was applied in 15-ml-sizing screw cap tube with 2 ml of absolute ethanol and 1 ml of 5.88 M KOH before vortex-mixing. Cap was tightly closed, and this tightened screw cap tube was boiled at 85 °C for 15 min before leaving to cool in ice bath immediately. 1.5 ml of hexane and 1 ml of deionised water were added and mixed for 1 min. 1 ml of hexane layer was applied to 2-ml-sizing glass vial tube and, subsequently, let to evaporation (Labconco, USA) at 40 °C for 30 min. 1 ml of 500 mg/L o-phthaldialdehyde (OPA) solution and 250 µl of concentrated sulphuric were added and mixed. The solution was rested for 20 min before measuring at 570 nm of spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA). Cholesterol content in supernatant and cell pellets was reported in term of percent (Pereira and Gibson 2002; Tomaro-Duchesneau et al. 2014; Yehia et al. 2015).

$$\%\ Residual\ cholesterol\ content\ in\ supernatant = \frac{A}{B} \times 100\%$$

$$\%\ Cholesterol\ content\ in\ cell\ pellets = \frac{C - D}{B} \times 100\%$$

$$\%\ Loss\ of\ cholesterol\ content = \frac{B - [A + (C - D)]}{B} \times 100\%$$

When A = cholesterol content in supernatant after incubation

B = cholesterol content in abiotic control set of LAB basal medium + cholesterol

C = cholesterol content in cell pellets after incubation

D = cholesterol content in cell pellets before incubation as control set

6. Investigation of γ-aminobutyric acid-producing LAB

6.1. Selection of γ-aminobutyric acid-producing LAB by TLC

Investigation of GABA by thin layer chromatography (TLC) was modified from (Choi et al. 2006; Hiraga et al. 2008). Contestant strains collected from secondary screening were allowed for γ-aminobutyric acid (GABA) investigation. After preparing the LAB strains from working cultures in 18 h of MRS broth incubation, 3% (v/v) inoculum contestant LAB was inoculated in 5 ml of MRS medium broth supplemented with 20 mg/ml monosodium glutamate (MSG) (Topic 8.1, Appendix A) and 5 ml of GYP medium broth supplemented with 20 mg/ml MSG (Topic 8.2, Appendix A). Temperature at 35 °C for 24 h was used for incubation. After finishing incubation, the culture supernatant was received from culture centrifugation at 5,000 × *g* for 10 min, and culture supernatant allowed for GABA testing by TLC (Topic 6.2, Appendix B). In brief, 3 µl of each sample was spot on TLC plate (Silica gel 60 F_{254}, Merck) before putting in the solvent chamber of butan-1-ol: glacial acetic acid: deionised water at 4: 1: 1. After finishing, TLC plate was dried before spraying the 1 mg/ml ninhydrin solution and incubated at 100 °C for 10 min for colouring. Retardation factor (R_f) was evaluated and the contestant LAB showing the GABA production selected from TLC was further estimated the amount of GABA production by high-performance liquid chromatography (HPLC).

6.2. Quantity estimation of GABA production by LAB using HPLC

Contestant strains were prepared in MRS medium broth for 18 h incubation at 35 °C. 3% (v/v) inoculum was inoculated in 10 ml of MRS broth supplemented with 5 mg/ml MSG and incubated at 35 °C for 72 h. The sample was collected every 24 h from 0 until 72 h. Culture supernatant was received by harvesting of culture at 5,000 × *g* for 10 min centrifugation and stored in -20 °C for waiting to estimate with HPLC. The condition to analyse glutamic acid and GABA contents was referred from (Populin et al. 2007) described in (Topic 7.2, Appendix B). Sententiously, HPLC tools (Agilent 1200 Series, Agilent, USA) combined with Hypersil ODS C_{18} (79926OD-584; Agilent, USA) sizing at 250 × 4.0 mm with 5 µm as particle size with 30 °C for temperature control. 15 µl of sample was derivatised with 75 µl of OPA

solution (Topic 7.1, Appendix B) before injection. Fluorescence detector (FLC) was used with 330 nm and 440 nm as excited and emitted wavelengths. Mobile phase content composed of 0.2 M phosphate buffer pH 7.0 and 100% acetonitrile with gradient following 87% at 0 - 15 min, 50% at 40 min, 15% at 60 – 62 min of phosphate buffer. Standard curves of both glutamic acid and GABA were constructed in a range of 0.1 – 8.0 mg/L.

After determination of glutamic acid and GABA concentrations of each sample, % conversion efficiency of MSG as substrate, GABA as product, total conversion and loss from this conversion were computed based on stoichiometric relationship.

7. Identification of selected LAB strains

7.1. Morphological characterisation

After knowing the selected strains for using in the experiment in advance, morphological characteristics were performed to educate these contestant strains including Gram's staining, bacterial cell shape, and bacterial arrangement.

7.2. Phenotypic characterisation

Phenotypic characterisation of selected LAB strains was consequent by biochemical estimation. To start with contestant LAB preparation cultured in MRS broth medium for 18 h, API 50 CHL (Ref 50410; BioMérieux, France) was authenticated for this testing that briefly described in (Topic 8.2, Appendix B). Concisely, 18 h of each contestant culture was twice washed with NSS before turbidity adjustment to 2 McFarlands was applied in API 50 CHL medium. 120 µl of each culture was meticulously put in the API 50 CHL strip before closing with 50 µl of paraffin oil. Results were read at 24 and 48 h of incubation at 35 °C and encrypted by apiweb™ identification software with database (V5.1) to identify the taxonomical appellation of those contestant LAB.

7.3. Genotypic characterisation

Six contestant strains were identified using 16S rRNA region. These strains were cultured from -80 °C cryopreservative in MRS broth for this testing. Procedures are shown (Topic 9.2, Appendix B). Briefly, DNA extraction was propelled by QIAamp® DNA mini kit (Qiagen, Germany). Genomic DNA was amplified the 16S rRNA region by HotStar HiFidelity Polymerase Kit (Qiagen, Germany) for 70 µl as total volume with composition including 3 µl of template, 14 µl of 5x nitrogenous base and magnesium sulphate buffer, 2.8 µl of 10 µM 27F primer as forward direction (5'-AGA GTT TGA TCC TGG CTC AG -3'), 2.8 µl of 10 µM 1492R primer as reverse direction (5'- GGT TAC CTT GTT ACG ACT T -3'), 0.84 µl of 2.5 units/ml HotStar HiFidelity polymerase and remaining volume of RNase-free water. Amplification condition was orderly set as 95 °C for 5 min of initial activation, 94 °C for 15 s of denaturation, 46.4 °C for 1 min of annealing, 72 °C for 1 min 45 s of extension and 72 °C for 10 min of final extension with 35 cycles from denaturing to extensive step. Purification of PCR product was applied by Gel and PCR purification system (BioFACT, South Korea) before the expected DNA was sequenced (1st Base, Malaysia). After receiving the information as base sequences, the sequences were applied in Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the most strain similarity of LAB before collecting companion LAB strains to construct a phylogenetic tree. Sequences were aligned with BioEdit version 7.6.2.1 (Hall 1999) and constructed by MEGA (Tamura et al. 2007). The tree was constructed to display contestant name and relationship by Bootstrap method of phylogeny with 1,000 replication tests in Jukes-Cantor substitution model in Neighbour-joining statistical method. An out-group was previewed by *Alloiococcus otitis* DSM7252^T (NR026088) to compare with contestant strains. Nucleotide sequences were accessed to NCBI database by Submission Portal (https://submit.ncbi.nlm.nih.gov/) only two important strains named ENM104 and SPS109 which were used in experiments in advance.

8. Kinetic growth of both selected LAB strains

8.1. Growth curve

Two LAB strains including ENM104 and SPS109 were selected for the further experiments. Thus, kinetic growth was necessitated to forecast some special characteristics of both contestants to use in various examinations. Both strains were prepared in 18 h of incubated MRS broth before examination. They were adjusted the turbidity with OD_{660} spectrophotometry at 1.0. A 3% (v/v) of inoculum was placed in 10 ml of MRS broth and incubated at 35 °C for the maximum time at 24 h. Sample was harvested at 0, 1, 2, 3, 4, 8, 12, 16, 20 and 24 h of incubation by turbidity measurement with spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA) and pH measurement (LAQUAtwin, Horiba, Japan). Triplicate needed for statistical calculation to construct the growth curve.

8.2. Comparison of cell numbers to cell turbidity

Both ENM104 and SPS109 were prepared from working culture for 18 h of incubated MRS broth medium. Bacteria were adjusted the turbidity with OD_{660} spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA) to 0.250, 0.500, 0.750, 1.000. Viable cells were counted with MRS agar by pour plate technique and incubated at 35 °C for 24 – 48 h. Relationship between absorbance OD_{660} and viable count was constructed. Triplicate needed for statistical analysis.

8.3. Viable count of cell growth at 18 h

Both contestant strains including ENM104 and SPS109 were prepared in 18 h of incubated MRS broth. Viable cells in MRS agar were used as a tool to this examination. Triplicate needed for statistical analysis.

9. Examination of probiotic properties of potent LAB strains

9.1. Hydrolysis of macromolecules

This examination was studied on the hydrolysis activity of contestant LAB for carbohydrate, protein, and lipid. To start this experiment, 18 h of incubation both contestant strains including ENM104 and SPS109 were prepared in MRS broth medium. 1 ml of each contestant cell culture was harvested by 5,000 × *g* centrifugation for 10 min and discarded the supernatant out. Cells were twice washed with NSS and finally also suspended in NSS. One loopful of each cell suspension was dropped on examined medium including 2 g/L starch agar, 20 ml/L skim milk agar and 10 ml/L tributyrin agar (Topic 9, Appendix A). Triplicate points of loopful needed for statistical estimation. A temperature at 35 °C for 24 and 48 h incubation was used for this test. Opalescent zone was investigated for hydrolysis activity as a positive result. Only starch agar medium examination after finishing the incubation was necessarily flooded by Lugol's iodine solution over agar surface to see transparent zone amongst bluish medium of starch hydrolysis as positive result. For the control, *Bacillus subtilis* was used as positive control; meanwhile, *Escherichia coli* was used as negative control.

9.2. Activity of bile salt hydrolases

Bile salts are constructed from cholesterol to use as a substrate for cholic acid derivatised into glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA) and taurodeoxycholic acid (TDCA). Probiotic LAB candidates usually produce bile salt hydrolyse to break bile salt to non-functionalised bile salt molecules to reduce the lipid transportation to human blood and lymph circulation. Both selected LAB including ENM104 and SPS109 were prepared in MRS broth medium and incubated at 35 °C for 18 h. One loopful of each strain was spot on MRS agar supplemented with 5 mg/ml of each bile salt including GCA, GDCA, TCA and TDCA (Topic 10, Appendix A) and incubated at 35 °C up to 72 h with reading the results every 24 h.

9.3. Estimation of gastrointestinal fluid tolerance *in vitro*

The tolerance of bacterial strains implementing in food industry is important to test the probiotic properties to resist the gastrointestinal conditions and survive after passing through the gastrointestinal tract. For this experiment, the contestant strains named ENM104 and SPS109 were used for this tolerant test as probiotic strains. As knowing about other probiotic properties including cholesterol-lowering level, health immunity association, and colonisation of probiotic strain to prevent pathogenic bacteria widespread, probiotic test was indispensably performed to confirm bacterial life survival through gastrointestinal tract to prolong and multiply themselves for generating benefits to host.

The procedure was modified (Ratanaburee et al. 2013b) with the following description as shown in the Figure 3-5. Both ENM104 and SPS109 contestant strains were firstly prepared in MRS broth from working culture and incubated 35 °C in 18 h. Second preparation was prepared by pipetting 5 ml of the first preparation culture to 500 ml MRS medium broth and incubating at 35 °C for 18 h. 500-ml MRS broth medium needed to harvest the final cell concentration roughly 10 log CFU/ml. Cells were harvested by centrifugation at 5,000 × *g* for 10 min and suspended in gastric fluid (Topic 12.1, Appendix A) and incubated at 37 °C for 2 h that was enough for digested time mimicking from stomach with microaerobic condition in screw cap test tubes. Cells before and after incubation were taken out for viable count with pour plate technique by MRS agar medium following the dilutions in the Table 3-1 with NSS. After ending of gastric fluid incubation, cells were removed by centrifugation at 5,000 × *g* for 10 min in 15 ml of conical plastic screw cap tube and, subsequently, poured out the gastric solution out. Intestinal fluid (Topic 12.2, Appendix A) was replaced immediately with similar volume and filled in the screw cap tube with microaerobic condition as well. The incubation was prolonged for 6 h simulated by the enrolment of diets to the intestinal tract. Cells were sampled out at 0, 3, and 6 h to measure viable cells as the above technique. Triplicate necessitated for statistical analysis.

Table 3-1. Viable count dilutions by saline solution

Gastrointestinal condition	Dilution fold
Gastric fluid 0 hour	10^8
Gastric fluid 2 hours	10^2, 10^3, 10^4,10^5
Intestinal fluid 0 hours	10^2, 10^3, 10^4,10^5
Intestinal fluid 3 hours	10^2, 10^3, 10^4,10^5
Intestinal fluid 6 hours	10^2, 10^3, 10^4,10^5

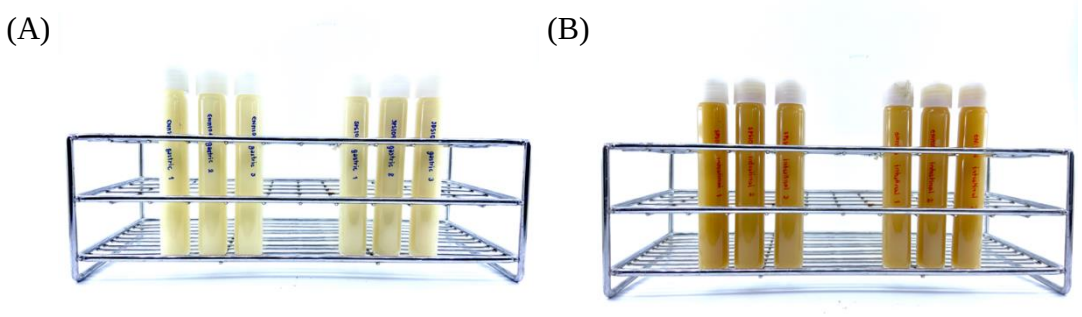


Figure 3-5. (A) Gastric fluid condition, (B) intestinal fluid condition of both strains testing as probiotic strains.

9.4. Property of cholesterol-lowering activity

From the cholesterol-lowering properties of ENM104 in LAB basal medium supplemented with 100 µg/ml cholesterol-PEG600 (water-soluble cholesterol), this experiment was performed in MRS broth with 100 µg/ml cholesterol-PEG600 to aim the % cholesterol-lowering content in MRS medium.

To begin with the activation of both contestant strains including ENM104 and SPS109, the stab culture stocked at 4 °C were streaked on MRS agar before incubation at 35 °C for 24 – 48 h. Single colony was inoculated in 10 ml of MRS broth medium and incubated at 35 °C for 18 h in preparation step. Each strain was adjusted depending on turbidity as OD_{660} at 1.0 to inoculate for 3% (v/v) inoculum in MRS broth supplemented with 100 µg/ml cholesterol-PEG600 (water-soluble cholesterol) as like as the Figure 3-6. Eight millilitres of volume size needed to perform microaerobic condition in 8 ml of sizing screw cap tube of each treatment and incubated

at 35 °C. Triplicate was allowed for statistical analysis. Treatments were sampled out at 0, 24, 48, and 72 h and every sample was managed to detect cholesterol content that was extracted by the modified method (Tomaro-Duchesneau et al. 2014) based on (Rudel and Morris 1973) to find % cholesterol-lowering content (Topic 3.3, Appendix B).

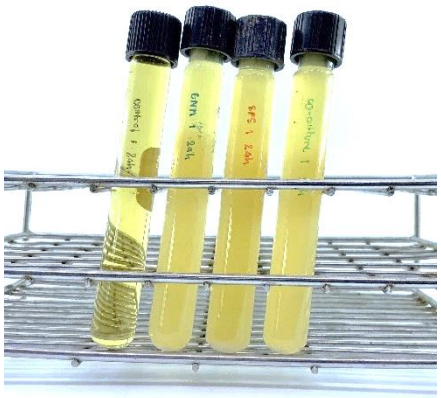


Figure 3-6. Property of cholesterol-lowering activity made up with 0, 24, 48 and 72 h fermentation time, respectively

9.5. Cholesterol oxidase assay

From the cholesterol-lowering properties of selected ENM104 strain in basal medium with 100 µg/ml cholesterol-PEG600, this experiment evaluated enzyme activity related to cholesterol degradation due to decrease amounts of cholesterol content. *P. pentosaceus* ENM104 was prepared by 10 ml of MRS broth cultivation from working culture for 18 h of incubation. 3% (v/v) of inoculum from OD_{660} at 1.0 was added in 50 ml of MRS broth supplemented with 100 µg/ml cholesterol (from 10 mg/ml cholesterol in ethanol) of screw cap tube and microaerobically incubated at 35 °C until 72 h with interval-collecting time at 24 h. Cell pellets and cell-free supernatants were collected depending on those times by centrifugation at 5,000 × *g* for 10 min at 4 °C of whole volumes in 50 ml-sizing conical tube. Cell-free supernatant was preserved at -20 °C until testing day; meanwhile, the whole of cell pellet was twice washed with 0.1 M phosphate buffer saline pH 7.4 and discarded all liquids out to preserve only cells at -20 °C as well. On the enzyme activity testing day with crude enzyme extract

preparation, cell pellets were suspended in 1.5 ml of deionised water and demolished with ultra-sonication (Sonics, USA) at 60% amplitude with 25 s and 5 s of on and off time for 10 min. Crude extracts were harvested by centrifugation at 12,000 × *g* for 10 min at 4 ℃ to eliminate cell debris out.

For the enzyme activity evaluation, procedure was subscribed (Ahire et al. 2012; Allain et al. 1974; Kiatpapan et al. 2001) with some adaptations for microtiter plate scale. Briefly, 200 µl of enzyme-reacting mixture composed of 64 mM of sodium cholate from bile salt no.3, 3.4 ml/L Triton X-100, 0.9 mM of cholesterol, 1.4 mM of 4-aminoantipyrine, 21 mM of phenol, and 1.5 U/ml of horseradish peroxidase were placed in a microtiter plate and incubated for 10 min at 37 ℃ for preparing of reaction. A 20 µl of crude enzyme extract was placed and immediately read with microplate reader (BMG Labtech, Germany) at 500 nm for 0 to 5 min. One unit of enzyme activity was provided by 1 µmol H_2O_2 per min. The regression range of H_2O_2 was stayed in the range of 0.2 – 20 nmol. Protein content was detected by Bradford method (Kruger 2003) with regression range of bovine serum albumin (BSA) as standard compound at 0.4 – 2.0 µg. Specific enzyme activity was defined from dividing of enzyme activity by protein content. Triplicate was used for statistical analysis.

10. Tolerance to hydrogen peroxide

Both contestant strains including ENM104 and SPS109 were prepared in MRS broth for 18 h of incubation. Cells were harvested by centrifugation at 5,000 × *g* for 10 min, twice washed with 0.1 M phosphate buffer saline (PBS; pH 7.4) and adjusted to roughly 8 log CFU/ml as inoculums. Control set was still suspended in PBS solution; meanwhile, treatment set was suspended in PBS supplemented with 1 mM hydrogen peroxide. Incubation at 35 ℃ needed for those testing with harvesting time at 0, 4, 8, 12, 16, 20 and 24 h. Viable cells were counted as a tool to detect the survival cell numbers in NSS and poured plate with MRS agar.

11. Determination of antioxidant activity and total phenolic content

11.1. Comparison of authentic compounds to determine antioxidant activity

Three authentic compounds were offered including L-ascorbic acid, gallic acid and trolox. These authentic compounds were compared amongst detected methods including 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobiz (3-ethylbenzothiazo line-6-sulfonic acid) (ABTS) and oxygen radical absorbance capacity (ORAC) (Banerjee et al. 2005; Ghiselli et al. 1995; Glazer 1990; Ou et al. 2001; Re et al. 1999). Standard concentrations of each authentic sample to perform authentic regression curves were used the range in the following. For both DPPH and ABTS method, L-ascorbic acid and trolox were made in 25 – 500 µM, while gallic acid was used in 10 – 200 µM. Furthermore, for ORAC method, L-ascorbic acid was done in 25 – 100 µM; meantime, trolox and gallic acid were performed in 10 – 40 µM. Procedure of three detected methods can be followed (Topic 10, Appendix B). Briefly for DPPH method, 20 µl of samples were mixed with 280 µl of 107.14 µM DPPH solution and waited for 30 min before detecting with 517 nm spectrophotometry by microplate reader (BMG Labtech, Germany); moreover, for ABTS, 20 µl of samples were mixed with 280 µl of 107.14 µM ABTS solution and waited for 3 min before detecting with 734 nm spectrophotometry by microplate reader (BMG Labtech, Germany). Besides, ORAC was measured by placing 20 µl of samples, 80 µl of 75 mM phosphate buffer pH 7.4 and 80 µl of 150 nM fluorescence solution. The reaction was started by adding 20 µl of 187.5 mM AAPH solution and immediately detected with fluorescence microplate reader (Colona Electric, Japan) at 480 nm and 520 nm for 60 min of excited and emitted wavelengths, respectively. Regression equation was outputted from this comparison with showing of regression curve. Triplicate was used as statistical analysis.

11.2. Antioxidant activity of selected LAB in MRS medium

Antioxidant activity was studied by both of contestant strains including ENM104 and SPS109 in MRS broth medium. Both strains were prepared by cultivation in MRS broth for 18 h of incubation. 3% (v/v) inoculum of OD_{660} was added in MRS broth of 8-ml-sizing screw cap tube. Incubation at 35 °C from 0 until 72 h with interval time at 24 h was performed as Figure 3-7. A 1.5 ml of each sample was centrifuged at

5,000 × *g* for 10 min to separate cell pellet out from cell-free supernatant. New microtube was used to collect the cell-free supernatant and stored at -20 °C refrigerator. On the experimental date, samples were meticulously throwed with not over than 35 °C. Various antioxidant activity method detections were used as tools to determine (Topic 10, Appendix B) including DPPH, ABTS and ORAC.

Figure 3-7. Experimental design for antioxidant activity estimation with 0, 24, 48 and 72 h of bacterial fermentations, respectively

11.3. Total phenolic content of both selected LAB

Procedure of this experiment was familiar with the estimation of antioxidant activity as the above. After throwing the samples with meticulously way, samples were tested to find the content of phenolic compounds with total phenolic content (TPC). Concisely, 20 µl of sample was placed in each microplate well and followed by 200 µl of water, 20 µl of 0.2 N Folin-Ciocalteu reagent and 60 µl of 75 g/L Na_2CO_3 for 30 min incubation before measuring with 765 nm spectrophotometry by microplate reader (BMG Labtech, Germany). Results were compared back with gallic acid standard curve to find content of phenolic compounds in term of gallic acid equivalent (µmol GE/ml sample).

12. Application of potent LAB strains as starter(s) for fermented dairy products

12.1. Fermented milk

This experiment aimed to use two selected LAB including ENM104 and SPS109 strains as starters either single or a mixed culture for milk fermentation with expecting to obtain functional fermented milk products. Both contestant strains were cultivated on MRS agar at 35 °C for 24 – 48 h for activation and inoculated one loopful of each culture into 10 ml of MRS broth and incubated at 35 °C for 18 h. Both bacterial cells were harvested from MRS broth by centrifugation at 5,000 × g for 10 min and twice washing with NSS. In the next, both bacterial cells were adjusted turbidity at 1.0 of OD_{660}. Four Duran's bottles containing of 200 ml of sterile whole milk were prepared (Topic 15, Appendix A) following the treatment conditions (details, Figure 3-8 and Figure 3-9).

1. Control set: only sterilised whole milk
2. ENM104 set: 3% inoculum of ENM104 single starter
3. SPS109 set: 3% inoculum of SPS109 single starter
4. Co-culture set: 1.5% inoculum of ENM104 combined with 1.5% inoculum of SPS109

A 200 ml of milk in each bottle was inoculated with inoculum size at 3% for individual set to provide a final cell density roughly 7 log CFU/ml, while a co-culture set using 1.5% of each strain to attain roughly 6.5 log CFU/ml. Then 15 ml was aseptically transferred into 16 ml of glass-screw cap tube to achieve microaerobic condition. The whole set of experiment was brought to incubate at 35 °C for 72 h. Samples were collected at 0, 24, 48 and 72 h of incubation time. Each sample at 5 ml was collected in 5 ml of microtube and stored in -20 °C until testing date. These following parameters: pH, acid content, bacterial viable count, cholesterol, GABA, glutamic acid and TPC contents, angiotensin-converting enzyme (ACE) inhibition and antioxidant activity were monitored.

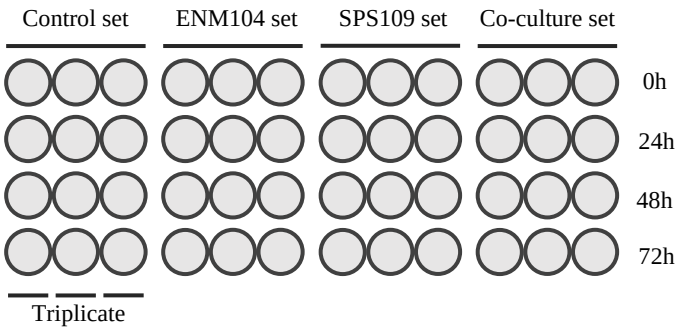


Figure 3-8. Experimental design for fermented dairy products by LAB starter(s)

Figure 3-9. Milk fermentation contributing of control, ENM104, SPS109 and co-culture sets, respectively

Several parameters were performed to detect in this experiment as previously described.

pH was immediately detected after arriving with each harvesting time by portable pH meter (pH-22, Horiba, Japan). Sample tubes were overwhelmingly mixed before sampling around 200 µl out to drop on the pH sensor.

Acid content with acid titration method (AOAC 2005) to find % acidity (Topic 12, Appendix B). Briefly, 1 ml of each sample was placed in 25 ml of carbon dioxide-eliminated distilled water with three drops of phenolphthalein solution and followed by titration with known concentration of sodium hydroxide (NaOH) solution which standardised with potassium hydrogen phthalate ($KHC_8H_4O_4$) (Topic 12.2, Appendix B) until the colourless turned to pinkish colour. Volume of NaOH solution was reported to stoichiometrically calculate for acid content.

Viable cell count was one of the various parameters whose immediate detection could be performed. Concisely, sample was diluted with NSS and appropriate dilutions i.e. 10^{-6} to 10^{-8} were used to count by pour plate technique using differentiated medium between *Pediococcus* and *Lactobacillus* (Topic 17, Appendix A) that modified (McDonald et al. 1987) for only this experiment. Incubation at 35 °C for 24 – 48 h needed for bacterial growth. White-pin-point colonies indicated *Pediococcus* sp., while *Lactobacillus* sp. was examined by yellowish colonies with yellowish zone around colonies.

The important parameter was cholesterol-lowering levels (Rudel and Morris 1973; Tomaro-Duchesneau et al. 2014). Briefly, the extraction step was familiar with cholesterol extraction in MRS broth fortified with cholesterol, but the whole sample was thoroughly mixed before sampling; however, after finishing the evaporation step, dry matter in HPLC vial was tightly closed and preserved in -20 °C until HPLC determination. For the HPLC condition, HPLC with diode array detector (DAD) was used for this determination with Nucleosil 100-5 C_{18} reversed phase (720014.40, 250 × 4.0 mm, 5 µm; Macherey-Nagel, Germany). Wavelength used at 205 nm with reference wavelength at 500 nm compromised with the bandwidth at 6 nm of both wavelengths. Thermostat was set up at 30 °C. Sample was dissolved by 500 µl of methanol spiked with 50 µg/ml of cholesterol standard and injection was used the volume at 50 µl to the mobile phase composed of acetonitrile: methanol: 2-propanol in the ratio at 7: 3: 1. Regression curve was used cholesterol standard in the range 0.2 – 200 µg/ml with spiked at 50 µg/ml.

Determination of GABA and glutamic acid quantification in milk samples was also determined. Milk samples were prepared to obtain extraction before injecting to determine with HPLC-FLD. To start with whey extraction (Abubakr et al. 2012) from milk samples (Topic 13, Appendix B), 1.2 ml of sample was taken by 1 M HCl to drop the pH down around pH 4.6. Centrifugation at 17,000 × *g* for 10 min was performed to separate casein-agglutinating part and whey supernatant out. A 0.22 µm nylon filter membrane was performed to filtrate the extract to be clear. This extraction step can be used in various detected parameters including of GABA and glutamic acid quantification and ACE inhibition. After milk extraction, HPLC-FLD (Populin et al. 2007) was performed as the previous procedure (Topic 7, Appendix B).

Extraction of antioxidative compounds was modified (Vázquez et al. 2015). In brief, 800 µl of milk sample was added and followed by 100 µl of MeOH:dIH_2O (1:1) mixture before adding 50 µl of Carrez I, 50 µl of Carrez II, 500 µl of acetonitrile. MeOH:dIH_2O mixture was added to make the volume to be full at 2,500 µl before centrifugation at 10,000 × g for 10 min was selected to separate. Supernatant parts were used to estimate antioxidant activity with three different methods including DPPH (Banerjee et al. 2005), ABTS (Re et al. 1999) and ORAC (Ghiselli et al. 1995; Glazer 1990) as previously described.

Detection of the efficiency of angiotensin-converting enzyme (ACE) inhibition was the one of various parameters that can be performed when finishing the whole experiment. Whey extraction step collected as the above procedure before examination. The whey parts were used for the ACE inhibition (Nakano et al. 2006) in the next (Topic 14, Appendix B). Concisely, 5 µl of whey-extracted sample was mixed with 25 µl of HEPES buffer and followed by 10 µl of 20 mU/ml ACE enzyme solution, 10 µl of ACE substrate solution. Incubation at 37 °C for 30 needed for ACE reaction. The reaction was stopped by adding 40 µl of 0.1 M NaOH. Liberated compounds from the reaction which was His-Leu was detected by adding 20 µl of OPA solution and incubating at room temperature for 30 min for binding reaction. The binding reaction was ended by adding 100 µl of 0.1 M HCl solution and incubating at 37 °C for 30 min. Complexed liberated His-Leu-OPA was measured by fluorescence spectrophotometer with 355 nm and 460 nm of excited and emitted wavelengths. The following formula was used to calculate inhibition: P as emission of positive control and S as emission of sample.

$$\%\ inhibition = \frac{P - S}{P} \times 100\%$$

12.2. Fermented whey

This experiment was designed familiar with the above experiment on milk fermentation as milk was replaced by whey (Figure 3-10). Whey preparation for this experiment was composed of several steps. In concise, 1 L of pasteurised milk purchased in convenient store was added by 2.5 g of commercial citric acid. To be clotted, milk was undisturbedly placed in water bath for 15 min at 85 °C for milk agglutination. Casein was separated by gauze and cotton to harvest only whey part. Whatman no.1 paper was finally used to make solution to be clear before stream-sterilisation at 121 °C for 15 min. Parameters to detect several activities with both selected strains fermentation were similar to milk fermentation.

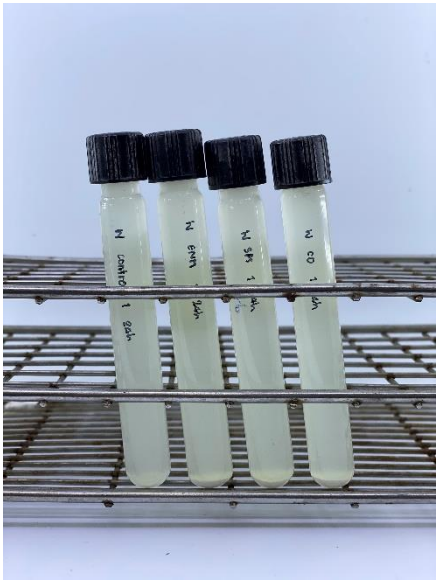


Figure 3-10. Whey fermentation contributing of control, ENM104, SPS109 and co-culture sets, respectively

13. Statistical analysis

Triplicate was necessitated for statistical analysis by one-way ANOVA of factorial analysis and some complexing experiments with two-way ANOVA of factorial analysis with significantly different analysis namely *post-hoc* test or multiple comparison by Tukey's HSD (honestly significant difference) at $p\text{-value} < 0.05$. R programme (version 3.5.2.) was used for analysed the above statistics.

CHAPTER 4

RESULT

1. Isolation and selection of LAB

Sixty samples of various fermented foods and raw milk (Table 4-1) collected from the many parts of Thailand found LAB in 39 samples (65 percent) with 244 LAB strains were isolated (Table 4-2).

2. Inspection of cholesterol-lowering LAB

With a total number of 244 isolated LAB, they were tested by primary screening for survival in the basal medium containing cholesterol and 0.1% (w/v) yeast extract. Then 139 LAB isolates were endured in the medium (Table 4-3) for secondary screening and found only 10 LAB isolates survived in the same culture medium but without yeast extract (Table 4-4). They were further tested using tertiary screening in the medium similar to primary screening and found that five strains including ENM104, ENM203, ENM204, NNM205 and NNM304 showed for higher viable cells amongst other strains. Moreover, three strains including ENM104, NNM205 and NNM304 proliferated their cells in treatment set with significant higher than control set ($p<0.05$) that showed in the range of 7.14 – 7.31 log CFU/ml in control set and 7.46 – 7.61 log CFU/ml in treatment set (Figure 4-1). Based on results, only three potent strains were led to the chemical evaluation of cholesterol use.

Table 4-1. Directory of fermented foods and raw milk samples

Date	Sample	Code	Collected location
03-09-2016	Hoi-Sieb (fermented shellfish)	SHS1	Sanam Chai, Sathing Phra, Songkhla
	Jing-jang (fermented fish)	SJJ1	
	Pang-daeng (pink fermented fish)	SPD1	
	Kung-som (fermented shrimp)	SKS1	Koh Yor, Mueang Songkhla, Songkhla
	Phung-pla-chon (fermented striped-snakehead fish)	SPP1	Khlong Dan, Ranot, Songkhla
	Phung-pla-kradi (fermented gouramis fish)	SPP2	
	Pla-duk-ra (fermented fish)	SPR1	
06-09-2016	Nham-mhu (fermented pork)	NNM1	Khun Nan, Chalerm Prakiat, Nan
	Pla-som (fermented fish)	NPS1	Chon Dan, Song Khwae, Nan
14-09-2016	Bu-du (fermented fish)	SBD1	Mueang Pattani, Pattani
19-09-2016	Ka-pi (shrimp paste)	SKP1	Nathab, Mueang Songkhla, Songkhla
	Pang-daeng (pink fermented fish)	SPD2	
23-09-2016	Nham-mhu (fermented pork)	ENM1	Mueang Mukdahan, Mukdahan
		ENM2	
		ENM3	
		ENM4	
	Som-mhu (fermented pork)	ESM1	
		ESM2	

Table 4-1. Continued

Date	Sample	Code	Collected location
24-09-2016	Bu-du (fermented fish)	SBD2	Khok Khiang, Mueang Narathiwat, Narathiwat
		SBD3	Paseyawor, Sai Buri, Pattani
	Pla-som (fermented fish)	SPS1	Kalisa, Ra Ngae, Narathiwat
25-09-2016	Pla-duk-ra (fermented fish)	SPR2	Don Sai, Khan Khanun, Phatthalung
05-10-2016	Ka-pi (shrimp paste)	SKP2	Tanyong Po, Mueang Satun, Satun
08-10-2016	Ka-pi (shrimp paste)	SKP3	Pak Phanang, Nakhon Si Thammarat
	Kung-som (fermented shrimp)	SKS2	
	Nhang-nhua (fermented beef)	SNG1	Khanab Nak, Pak Phanang, Nakhon Si Thammarat
16-10-2016	Pla-prew (fermented fish)	SPS2	Bang Muang, Ta Kua Pa, Pang Nga
23-10-2016	Pla-duk-ra (fermented fish)	SPR3	Mueang Nakhon Si Thammarat, Nakhon Si Thammarat
28-10-2016	Pla-duk-ra (fermented fish)	SPR4	Khao Chaison, Khao Chaison, Phatthalung
02-11-2016	Kung-som (fermented shrimp)	NKS1	Mueang Chiangmai, Chiangmai
	Nham-mhu (fermented pork)	NNM2	
		NNM3	
	Pla-ra (fermented fish)	NPR1	
		NPR2	
	Pla-som (fermented fish)	NPS2	
	Som-jin-mak (fermented fish)	NSJ1	

Table 4-1. Continued

Date	Sample	Code	Collected location
14-11-2016	Jing-jang (fermented fish)	SJJ2	Mueang Satun, Satun
	Kung-som (fermented shrimp)	SKS3	
	Pang-daeng (pink fermented dish)	SPD3	
14-12-2016	Jing-jang (fermented fish)	SJJ3	Kho Hong, Hat Yai, Songkhla
		SJJ4	Khlong Hae, Hat Yai, Songkhla
	Kung-som (fermented shrimp)	SKS4	Kho Hong, Hat Yai, Songkhla
		SKS5	Khlong Hae, Hat Yai, Songkhla
	Nham-kai (fermented chicken)	SNM1	Kho Hong, Hat Yai, Songkhla
		SNM2	
	Nham-pla (fermented fish)	SNM3	
		SNM4	
	Pang-daeng (pink fermented fish)	SPD4	
		SPD5	Khlong Hae, Hat Yai, Songkhla
	Phung-pla (fish belly)	SPP3	Khlong Hae, Hat Yai, Songkhla
	Pla-ra (fermented fish)	SPR5	
	Pla-som (fermented fish)	SPS3	

Table 4-1. Continued

Date	Sample	Code	Collected location
17-01-2017	Raw cow milk	ECM1	Nong Sarai, Pak Chong, Nakhon Ratchasima
		ECM2	
		SCM1	Khao Chaison, Khao Chaison, Phatthalung
		SCM2	
		SCM3	Pa Bon, Phatthalung
		SCM4	Khao Chaison, Khao Chaison, Phatthalung
	Raw goat milk	SGM1	Mueang Yala, Yala
		SGM2	

Date is orderly written as day-month-year. The detail of collected location is orderly composed by sub-district, district, and province as writing order of the Kingdom of Thailand.

Table 4-2. Directory of LAB isolated from fermented foods and raw milk

Code	Directory of isolate									
ECM1	ECM101	ECM102	ECM103	ECM104	ECM105	ECM106	ECM107	ECM108	ECM109	
ECM1XB	ECM101XB		ECM102XB							
ECM2	ECM201		ECM203		ECM205	ECM206				
ECM2XB	ECM201XB									
ENM1	ENM101	ENM102	ENM103	ENM104	ENM105	ENM106	ENM107	ENM108	ENM109	ENM110
	ENM111	ENM112	ENM113	ENM114	ENM115					
ENM2	ENM201	ENM202	ENM203	ENM204	ENM205	ENM206	ENM207	ENM208	ENM209	
	ENM211	ENM212								
ENM3	ENM301	ENM302	ENM303	ENM304	ENM305	ENM306	ENM307	ENM308	ENM309	ENM310
	ENM311	ENM312								
ENM4	ENM401	ENM402	ENM403	ENM404	ENM405	ENM406	ENM407	ENM408	ENM409	ENM410
ESM1	ESM101	ESM102	ESM103	ESM104	ESM105	ESM106	ESM107	ESM108	ESM109	ESM110
	ESM111	ESM112								
ESM2	ESM201	ESM202	ESM203	ESM204	ESM205	ESM206	ESM207	ESM208		ESM210
	ESM211									
NKS1	NKS101	NKS102	NKS103	NKS104	NKS105	NKS106				
NNM1	NNM101	NNM102	NNM103							
NNM2	NNM201	NNM202	NNM203	NNM204	NNM205	NNM206	NNM207	NNM208	NNM209	NNM210
	NNM211	NNM212								

Table 4-2. Continued

Code	Directory of isolate									
NNM3		NNM302	NNM303	NNM304	NNM305	NNM306	NNM307	NNM308	NNM309	NNM310
	NNM311									
NPR1	NPR101	NPR102								
NPR2	NPR201	NPR202	NPR203	NPR204	NPR205	NPR206	NPR207	NPR208		
NPS1	NPS101	NPS102								
NPS2	-									
NSJ1	NSJ101	NSJ102	NSJ103	NSJ104	NSJ105	NSJ106	NSJ107	NSJ108	NSJ109	NSJ110
	NSJ111	NSJ112	NSJ113	NSJ114	NSJ115	NSJ116				
SBD1	-									
SBD2	-									
SBD3	-									
SCM1	SCM101		SCM103	SCM104	SCM105	SCM106				
SCM2		SCM202	SCM203							
SCM3	SCM301	SCM302	SCM303	SCM304	SCM305	SCM306				
SCM4	SCM401	SCM402	SCM403							
SCM1XB	SCM101XB									
SCM2XB	SCM201XB									
SCM3XB	SCM301XB									

Table 4-2. Continued

Code	Directory of isolate						
SCM4XB	SCM401XB						
SGM1	SGM101	SGM102					
SGM2	SGM201						
SHS1	-						
SJJ1	-						
SJJ2	SJJ201	SJJ202	SJJ203				
SJJ3	-						
SJJ4	-						
SKP1	SKP201		SKP203	SKP204	SKP205	SKP206	SKP207
SKP2	-						
SKP3	-						
SKS1	SKS101						
SKS2	-						
SKS3	SKS301	SKS302	SKS303	SKS304			
SKS4	-						
SKS5	-						
SNG1	SNG101	SNG102		SNG104		SNG106	
SNM1	SNM101	SNM102		SNM104	SNM105	SNM106	

Table 4-2. Continued

Code	Directory of isolate									
SNM2	SNM201	SNM202		SNM204	SNM205	SNM206				
SNM3	SNM301		SNM303	SNM304	SNM305	SNM306				
SNM4	SNM401	SNM402	SNM403							
SPD1	-									
SPD2	-									
SPD3	SPD301	SPD302	SPD303							
SPD4	SPD401	SPD402	SPD403	SPD404	SPD405					
SPD5	-									
SPP1	-									
SPP2	-									
SPP3	-									
SPR1	SPR101	SPR102	SPR103	SPR104	SPR105					
SPR2	-									
SPR3	SPR301	SPR302	SPR303	SPR304	SPR305	SPR306	SPR307	SPR308		
SPR4	SPR401	SPR402	SPR403							
SPR5	-									
SPS1	SPS101	SPS102	SPS103	SPS104		SPS106	SPS107	SPS108	SPS109	SPS110
	SPS111	SPS112								
SPS2	SPS201	SPS202	SPS203	SPS204	SPS205	SPS206	SPS207			
SPS3		SPS302	SPS303	SPS304	SPS305	SPS306				

Dash (-) means no data available.

Table 4-3. Directory of primary screening survival LAB contestants in LAB basal medium supplemented with 100 μg/ml cholesterol and 0.1% (w/v) yeast extract

Code	Directory of primary screening survival contestant									
ECM1	ECM101	ECM102	ECM104	ECM105	ECM106	ECM107	ECM108	ECM109		
ECM1XB	ECM101XB		ECM102XB							
ECM2	ECM201	ECM203	ECM206							
ECM2XB	ECM201XB									
ENM1	ENM102	ENM103	ENM104	ENM105	ENM108	ENM109	ENM110	ENM112	ENM113	ENM114
ENM2	ENM201	ENM202	ENM203	ENM204	ENM205	ENM207	ENM208	ENM209		
ENM3	ENM303	ENM306	ENM307	ENM308	ENM309	ENM311	ENM312			
ENM4	ENM401	ENM402	ENM403	ENM405	ENM406	ENM407	ENM408	ENM409		
ESM1	ESM102	ESM104	ESM105	ESM106	ESM109	ESM111	ESM112			
ESM2	ESM210	ESM211								
NNM1	NNM101	NNM102	NNM103							
NNM2	NNM203	NNM204	NNM205	NNM206	NNM207	NNM208	NNM210	NNM211	NNM212	
NNM3	NNM303	NNM304								
NPR1	NPR101	NPR102								
NPR2	NPR204	NPR206	NPR207	NPR208						
NSJ1	NSJ102	NSJ103	NSJ104	NSJ108	NSJ110	NSJ113	NSJ116			
NPS1	NPS101	NPS102								
SCM1	SCM101	SCM105	SCM106							
SCM2	SCM202	SCM203								

Table 4-3. Continued

Code	Directory of primary screening survival contestant						
SCM3	SCM302	SCM303	SCM304	SCM305	SCM306		
SCM4	SCM401	SCM402	SCM403				
SCM1XB	SCM101XB						
SCM2XB	SCM201XB						
SCM3XB	SCM301XB						
SCM4XB	SCM401XB						
SJJ2	SJJ202	SJJ203					
SKP2	SKP201	SKP204	SKP205	SKP206	SKP207		
SKS1	SKS101						
SNG1	SNG104	SNG106					
SNM1	SNM101	SNM102	SNM104	SNM105			
SNM2	SNM202	SNM204	SNM206				
SNM3	SNM301	SNM303	SNM304	SNM305	SNM306		
SNM4	SNM402						
SPD3	SPD303						
SPR1	SPR102	SPR103					
SPR4	SPR401	SPR402	SPR403				
SPS1	SPS101	SPS103	SPS104	SPS109	SPS110	SPS111	SPS112
SPS2	SPS205	SPS206					

Table 4-4. Directory of secondary screening survival LAB contestants in LAB basal medium supplemented with 100 µg/ml cholesterol without yeast extract

Code	Directory of secondary screening survival contestant
ECM1XB	ECM101XB
ENM1	ENM104
ENM2	ENM203 ENM204
ENM4	ENM407
NNM2	NNM205
NNM3	NNM304
SCM3XB	SCM301XB
SCM4XB	SCM401XB
SPS1	SPS109

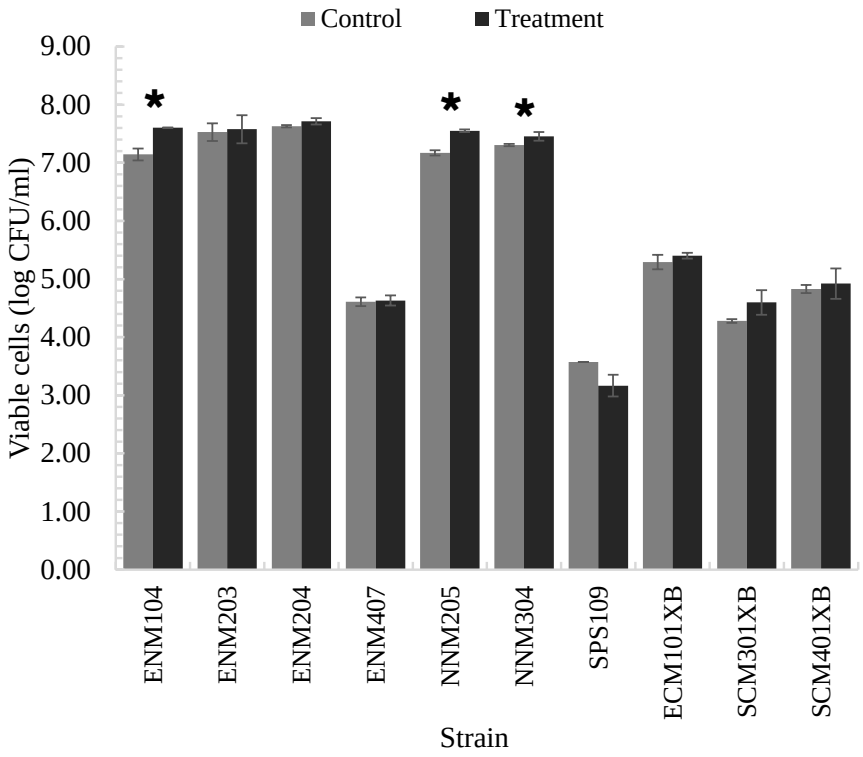


Figure 4-1. Comparison of LAB population found in tertiary screening between (■) control and (■) treatment, asterisk (✱) representing for significant differences ($p<0.05$) between control and treatment proliferation

3. Use of cholesterol by potent LAB

Table 4-5 shows results that received from the cholesterol-PEG600 regression equation representing y = 0.0034x + 0.0045 with R^2 = 0.9977 (Topic 3, Appendix B) with the cholesterol content in abiotic control set at 125.13 ± 1.72 µg/ml that was tiny higher than the theoretical planning from 100 µg/ml. ENM104 strain displayed a good result of cholesterol reduction in culture supernatant after finishing fermentation at 115.36 ± 3.83 µg/ml and cell absorption at 0.35 ± 0.07 µg/ml cholesterol with the significant highest cholesterol loss at 9.43 ± 2.23 µg/ml. To be more understanding previewing in the Figure 4-2, ENM104 strain showed the proportion in culture supernatant, cell pellet, and cholesterol loss at 92.19 ± 3.06, 0.28 ± 0.05 and 7.53 ± 1.78 percent, respectively.

Table 4-5. Cholesterol content (µg/ml) from the spectrophotometry detection calculated by stoichiometry

Strain	**Cholesterol content (µg/ml)**		
	Intracellular	**Extracellular**	**Loss**
ENM104	0.35 ± 0.07 ^{e}	115.36 ± 3.83 ^{b}	9.43 ± 2.23 ^{a}
ENM203	0.87 ± 0.36 ^{d}	121.70 ± 1.86 ^{a}	2.57 ± 0.98 ^{b}
ENM204	1.69 ± 0.48 ^{c}	121.89 ± 4.55 ^{a}	1.56 ± 1.47 ^{d}
NNM205	2.91 ± 0.46 ^{a}	120.32 ± 4.14 ^{ab}	1.90 ± 0.42 ^{cd}
NNM304	2.37 ± 0.42 ^{b}	120.68 ± 3.39 ^{ab}	2.08 ± 1.87 ^{bc}

Abiotic control cholesterol contents = 125.13 ± 1.72 µg/ml and different lowercase alphabets representing as significant differences ($p<0.05$).

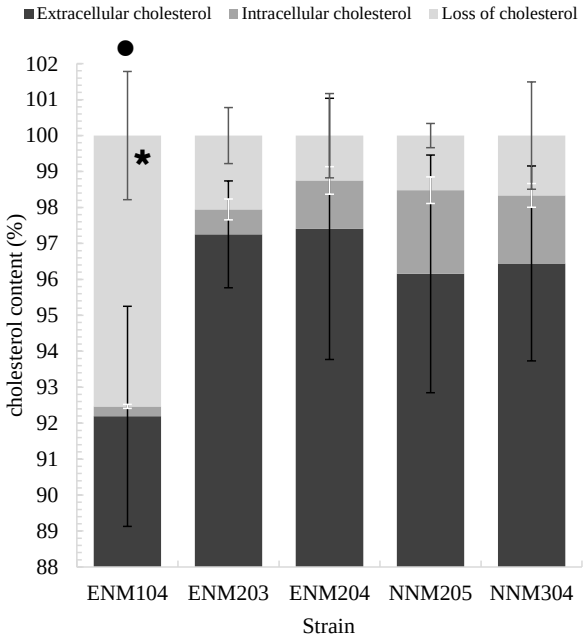


Figure 4-2. The proportion of cholesterol content amongst five LAB strains with the percentage of cholesterol loss (▒), the percentage of intracellular cholesterol (▒), the percentage of extracellular cholesterol (■), and the percentage of abiotic control (—) , and (✱) representing the significant highest result ($p<0.05$) of cholesterol loss compared with the other strain results and (●) representing the best LAB strain

4. Investigation of γ-aminobutyric acid-producing LAB

Ten LAB isolates were chosen from secondary screening to spot on TLC for γ-aminobutyric acid (GABA) investigation. Then this led to the selection of GABA-producing strains, and results were displayed in the Figure 4-3. Only SPS109 strain showed GABA production with this preliminary step testing by R_f at 0.32 as GABA; however, all tested LAB strains displayed R_f at 0.20 as MSG. For the quantified estimation of GABA and glutamic acid by HPLC, concentration of MSG and GABA were previewed in the Figure 4-4, while the percentage of conversion efficiency was displayed in the Table 4-6. GABA was produced from this conversion for 37.73 ± 0.10% with the significantly highest content at 72 h of incubation ($p<0.05$); moreover, mass lost from this conversion was found in slight declining. By the way, initial concentration of MSG from stoichiometric calculation of glutamic acid detected by HPLC was 5,028.50 ± 7.48 µg/ml that was higher than MSG containing in MRS medium at 5,000 µg/ml by 0.57 ± 0.15 percent.

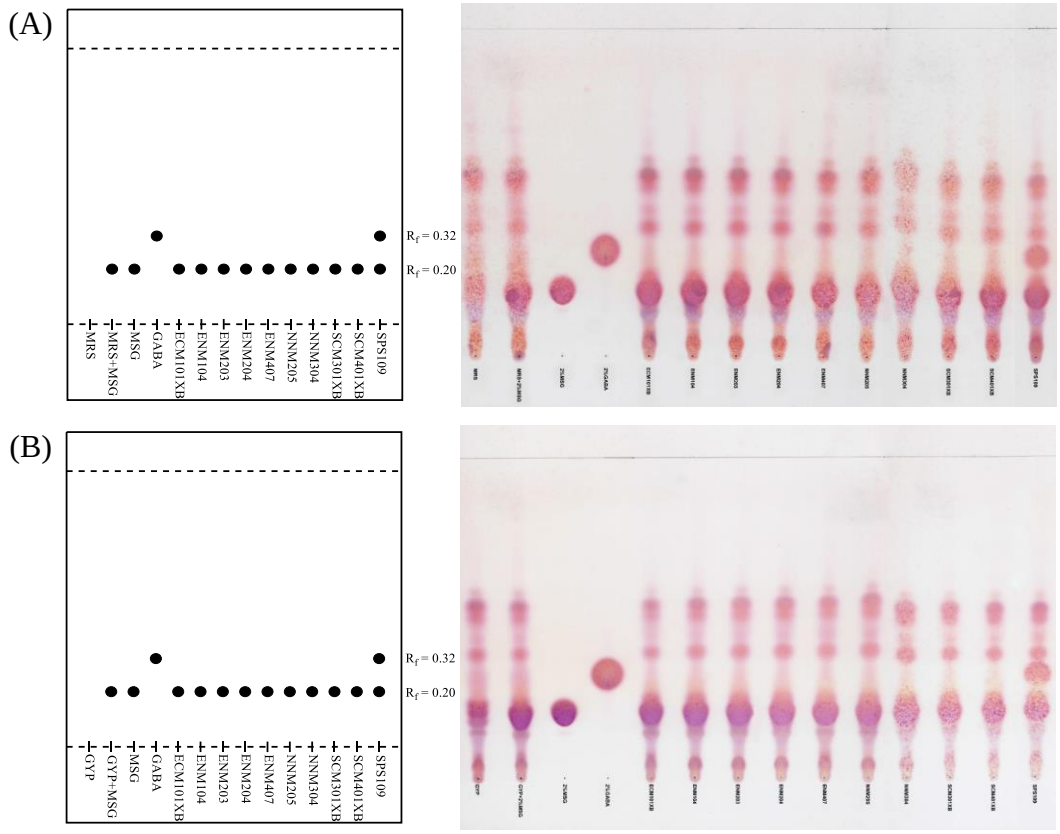


Figure 4-3. Investigation of GABA production by LAB using TLC; (A) TLC with MRS culture supernatant, (B) TLC with GYP culture supernatant by the representing position including MRS/GYP as medium control, MRS/GYP + MSG as MSG control, MSG and GABA as standard substances, ECM101XB, ENM104, ENM203, ENM204, ENM407, NNM205, NNM304, SCM301XB, SCM401XB, SPS109 as contestant LAB strains

Table 4-6. The percentage of conversion efficiency from MSG to GABA in MRS medium supplemented with 5 mg/ml MSG

Incubation time (h)	**% Conversion efficiency**			
	MSG	**GABA**	**Total**	**Loss**
0	100.00 ± 0.00 [a]	0 [d]	100.00 ± 0.00 [a]	0 [b]
24	81.46 ± 0.26 [b]	15.07 ± 0.15 [c]	96.53 ± 0.41 [b]	3.47 ± 0.41 [a]
48	66.41 ± 0.29 [c]	29.74 ± 0.58 [b]	96.16 ± 0.86 [b]	3.84 ± 0.86 [a]
72	61.59 ± 0.04 [d]	37.73 ± 0.10 [a]	99.33 ± 0.14 [a]	0.67 ± 0.14 [b]

Different lowercase letters are provided for significant differences depending on incubation time ($p<0.05$)

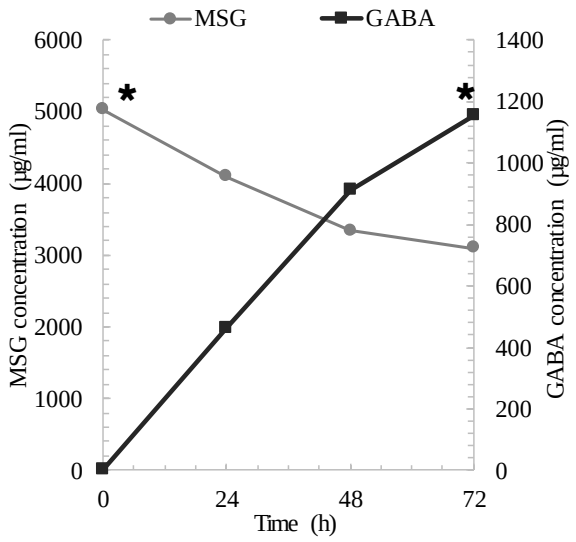


Figure 4-4. GABA (■) and MSG concentration (●) of SPS109 strain in MRS broth supplemented with 5 mg/ml MSG and (✱) representing significant differences with different incubation times ($p<0.05$)

5. Identification of selected LAB strains

With the morphological characteristics, Table 4-7 previews the results of morphological characteristics of six contestant strains from bulk bacterial isolation with Gram's staining, cell shapes and arrangements. Mostly strains excepted SPS109 were shown in cocci shape with the arrangement similar to tetrads, while SPS109 strain differed out that showed rod shape with the arrangement as chains. For the sampling source of these LAB isolates described by location and sampled foods, strains named ENM104, ENM203 and ENM204 were isolated from Traditional Thai fermented pork or Nham collected from the north-eastern region of Thailand. While NNM205 and NNM304 were isolated from Nham collected from the northern region of Thailand. For the SPS109, it was similar with *Lactobacillus* sp. which was isolated from Pla-som in the southern region of Thailand.

For strain identification, Table 4-8 previews the results of sugar fermentation from API 50 CHL test kit for the benefit of biochemical identification. With the characteristic of difference sugar usage, it can categorise contestant LAB strains for two groups. The first group can utilise D-mannitol, D-sorbitol, Methyl-αD-mannopyranoside, D-melibiose, D-melezitose, D-raffinose and potassium gluconate

representing for SPS109 strain, while the other group can utilise D-tagatose representing for the other five strains. Sugar information was analysed to distinguish for performing of the percentage of similarity as shown in the Table 4-9.

Furthermore, 16S rRNA technique was used to consider in this identification that showed gel electrophoresis of PCR products of six strains (Figure 4-5). All sample PCR products in the figure showed DNA bands tiny-over than 1,500 bp because of electrophoresis gradient effect. After sequencing, bioinformatics of 16S rDNA lengths were proceeded at 1,445 (ENM104), 1,449 (SPS109), 1,450 (ENM203), 1,426 (ENM204), 1,440 (NNM205), and 1,450 (NNM304) bp (Appendix C). The percentage of similarity was gathered from DNA base comparison (Table 4-10). Moreover, taxonomical characteristic is used as a comparison tool between two closet strains as shown in the Figure 4-6. SPS109 strain locates in *Lactobacillus* group, which is separated out from the other five strains which locate in *Pediococcus* group.

It was massively concluded that the six contestant strains including ENM104, ENM203, ENM204, NNM205 and NNM304 were identified as *P. pentosaceus* and SPS109 was *L. plantarum* with the % similarity over than 99 percent. For the further experiment, only *P. pentosaceus* ENM104 (Accessed number: MN915130) which is the % similarity at 99.65 to DSM20336^{T} (NR042058), and *L. plantarum* SPS109 (Accessed: number MN915131) that has the % similarity at 99.93 to DSM20174^{T} (NR115605) were selected for further examinations.

Table 4-7. Morphological characteristics of six contestant LAB strains

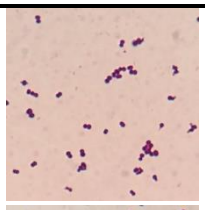
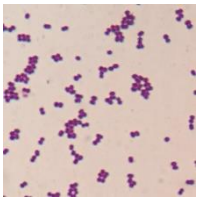
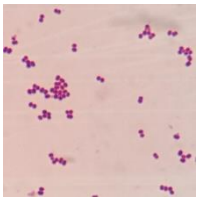
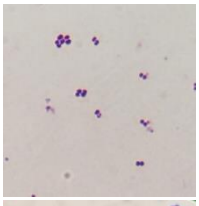
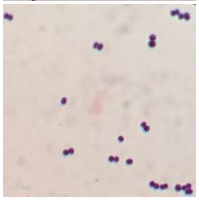
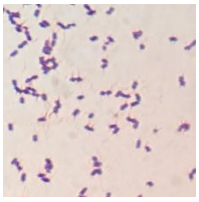
Strain	**Illustration**	**Morphological characteristic**		
		Gram's staining	**Shape**	**Arrangement**
ENM104		Violet; positive	Cocci	Tetrads
ENM203		Violet; positive	Cocci	Tetrads
ENM204		Violet; positive	Cocci	Tetrads
NNM205		Violet; positive	Cocci	Tetrads
NNM304		Violet; positive	Cocci	Tetrads
SPS109		Violet; positive	Rod	Pair or short chain

Table 4-8. Biochemical test of six LAB isolates by API 50 CHL (BioMérieux, France)

Well number	Carbohydrate test		LAB isolate					
	Ingredient	(mg/well)	ENM104	ENM203	ENM204	NNM205	NNM304	SPS109
0	Control	-	-	-	-	-	-	-
1	Glycerol	1.64	-	+	+	+	+	-
2	Erythritol	1.44	-	-	-	-	-	-
3	D-Arabinose	1.4	-	-	-	-	-	-
4	L-Arabinose	1.4	+	+	+	+	+	+
5	D-Ribose	1.4	+	+	+	+	+	+
6	D-xylose	1.4	+	-	-	+	+	-
7	L-xylose	1.4	-	-	-	-	-	-
8	D-Adonitol	1.36	-	-	-	-	-	-
9	Methyl-βD-xylopyranoside	1.28	-	-	-	-	-	-
10	D-Galactose	1.4	+	+	+	+	+	+
11	D-Glucose	1.56	+	+	+	+	+	+
12	D-Fructose	1.4	+	+	+	+	+	+
13	D-Mannose	1.4	+	+	+	+	+	+
14	L-Sorbose	1.4	-	-	-	-	-	-
15	L-Rhamnose	1.36	-	-	-	-	-	-
16	Dulcitol	1.36	-	-	-	-	-	-
17	Inositol	1.4	-	-	-	-	-	-
18	D-Mannitol	1.36	-	-	-	-	-	+
19	D-Sorbitol	1.36	-	-	-	-	-	+
20	Methyl-αD-mannopyranoside	1.28	-	-	-	-	-	+
21	Methyl-αD-glucopyranoside	1.28	-	-	-	-	-	-
22	N-Acetylglucosamine	1.28	+	+	+	+	+	+
22	Amygdalin	1.08	+	+	+	+	+	+
24	Arbutin	1.08	+	+	+	+	+	+
25	Esculin (+Ferric citrate)	1.16 (+0.152)	+	+	+	+	+	+
26	Salicin	1.04	+	+	+	+	+	+
27	D-Cellobiose	1.32	+	+	+	+	+	+
28	D-Maltose	1.4	+	+	+	+	+	+
29	D-Lactose (bovine origin)	1.4	-	-	-	+	+	+
30	D-Melibiose	1.32	-	-	-	-	-	+
31	D-Saccharose (sucrose)	1.32	-	+	-	+	+	+

Table 4-8. Continued

Well number	Carbohydrate test		LAB isolate					
	Ingredient	(mg/well)	ENM104	ENM203	ENM204	NNM205	NNM304	SPS109
32	D-Trehalose	1.32	+	+	+	+	+	+
33	Inulin	1.28	-	+	+	+	+	-
34	D-Melezitose	1.32	-	-	-	-	-	+
35	D-Raffinose	1.56	-	-	-	-	-	+
36	Amidon (starch)	1.28	-	-	-	-	-	-
37	Glycogen	1.28	-	-	-	-	-	-
38	Xylitol	1.4	-	-	-	-	-	-
39	Gentiobiose	0.5	+	+	+	+	+	+
40	D-Turanose	1.32	-	-	-	-	-	-
41	D-Lyxose	1.4	-	-	-	-	-	-
42	D-Tagatose	1.4	+	+	+	+	+	-
43	D-Fucose	1.28	-	-	-	-	-	-
44	L-Fucose	1.28	-	-	-	-	-	-
45	D-Arabitol	1.4	-	-	-	-	-	-
46	L-Arabitol	1.4	-	-	-	-	-	-
47	Potassium gluconate	1.84	-	-	-	-	-	+
48	Potassium 2-ketogluconate	2.12	-	-	-	-	-	-
49	Potassium 5-ketogluconate	1.8	-	-	-	-	-	-

Positive result (+) shows for yellowish colour; meanwhile, negative result (-) shows unchanged colour or purplish colour.

Table 4-9. Biochemical identification of six LAB isolates by apiweb™ identification software with database (V5.1)

Isolate	**LAB identification**	**% Similarity**
ENM104	*Pediococcus pentosaceus* 1	99.9
ENM203	*Pediococcus pentosaceus* 1	92.6
ENM204	*Pediococcus pentosaceus* 1	99.9
NNM205	*Lactobacillus brevis* 1	47.7
	Lactococcus lactis spp. lactis 1	38.9
	Pediococcus pentosaceus 2	10.0
	Pediococcus pentosaceus 1	3.1
NNM304	*Lactobacillus brevis* 1	47.7
	Lactococcus lactis spp. lactis 1	38.9
	Pediococcus pentosaceus 2	10.0
	Pediococcus pentosaceus 1	3.1
SPS109	*Lactobacillus plantarum* 1	99.9
	Lactobacillus pentosus	0.1

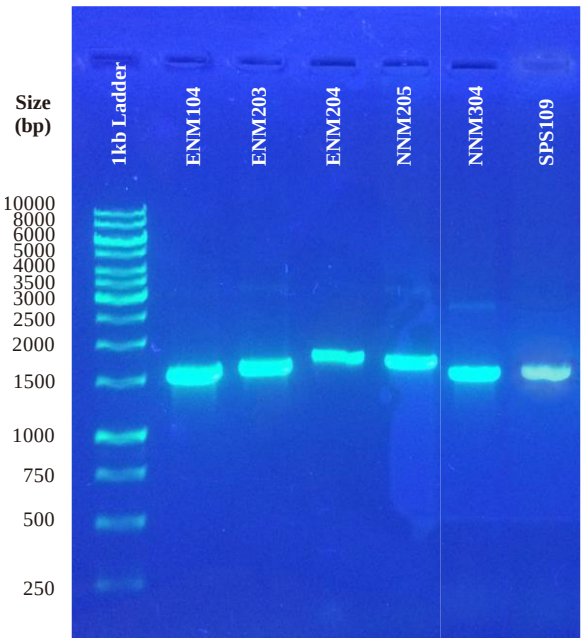


Figure 4-5. DNA examination by gel electrophoresis including DNA ladder Gene Ruler 1 kb DNA Ladder (Thermo Scientific, 2018) and contestant LAB isolates including ENM104, ENM203, ENM204, NNM205, NNM304 and SPS109, respectively

Table 4-10. The percentage of similarity of six contestant LAB strains

Isolate	**Identification result**	**% Similarity**
ENM104	*Pediococcus pentosaceus*	99.65
ENM203	*Pediococcus pentosaceus*	99.03
ENM204	*Pediococcus pentosaceus*	99.51
NNM205	*Pediococcus pentosaceus*	99.23
NNM304	*Pediococcus pentosaceus*	99.51
SPS109	*Lactobacillus plantarum*	99.93

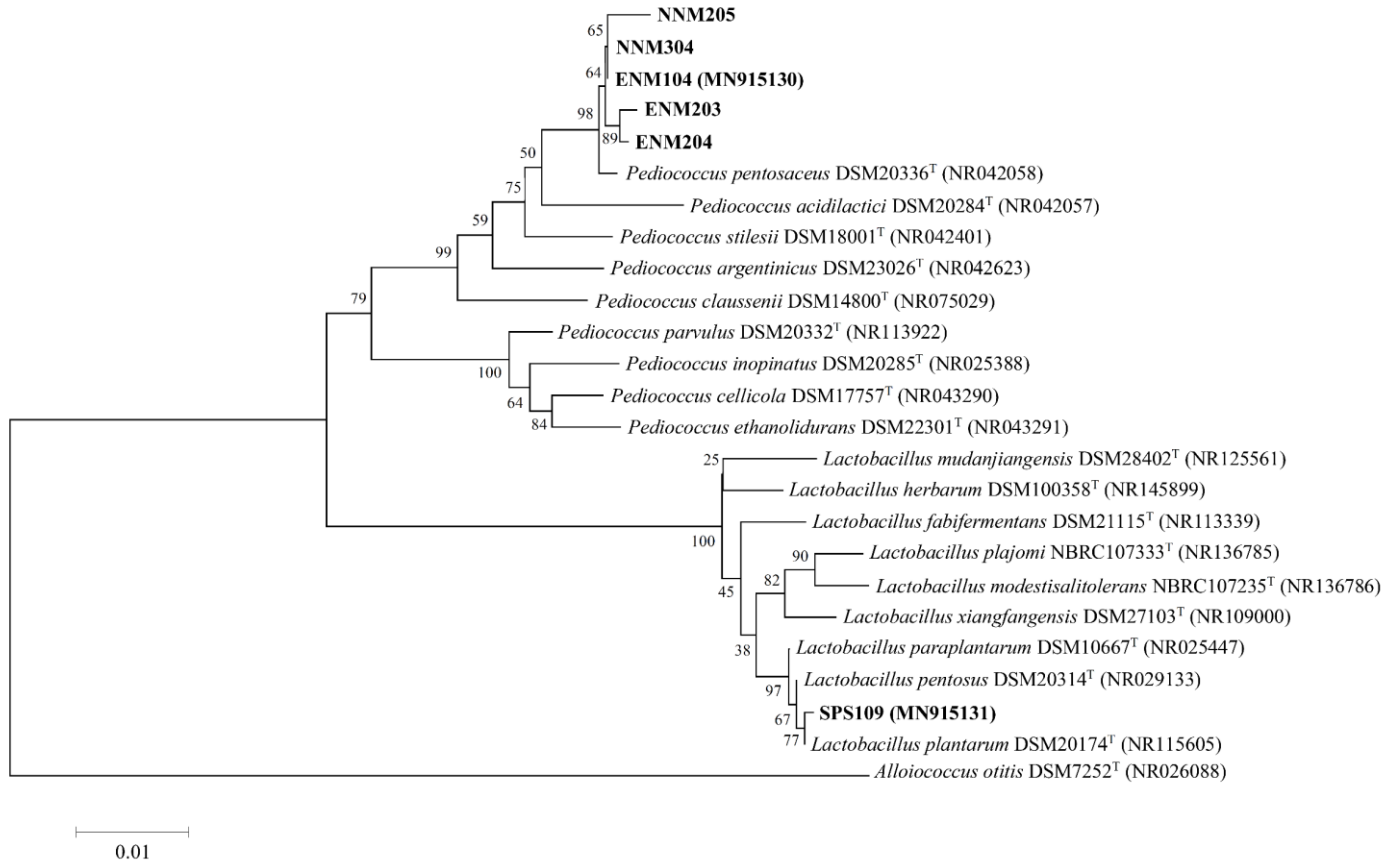


Figure 4-6. A phylogenetic tree of six candidate LAB isolates including ENM104, ENM203, ENM204, NNM205, NNM304 and SPS109 was operated by 16S rRNA identification and established with Jukes-Cantor nucleotide model of neighbour-joining statistical method. Annotation in the tree was comprised of genus, specific epithet, strain identifier, and accessing number in the parenthesis. An out-group was *Alloiococcus otitis* DSM7252^{T} (NR026088). Evolutionary distance was displayed by bar ratio representing by bar = 0.01.

6. Kinetic growth of both selected LAB strains

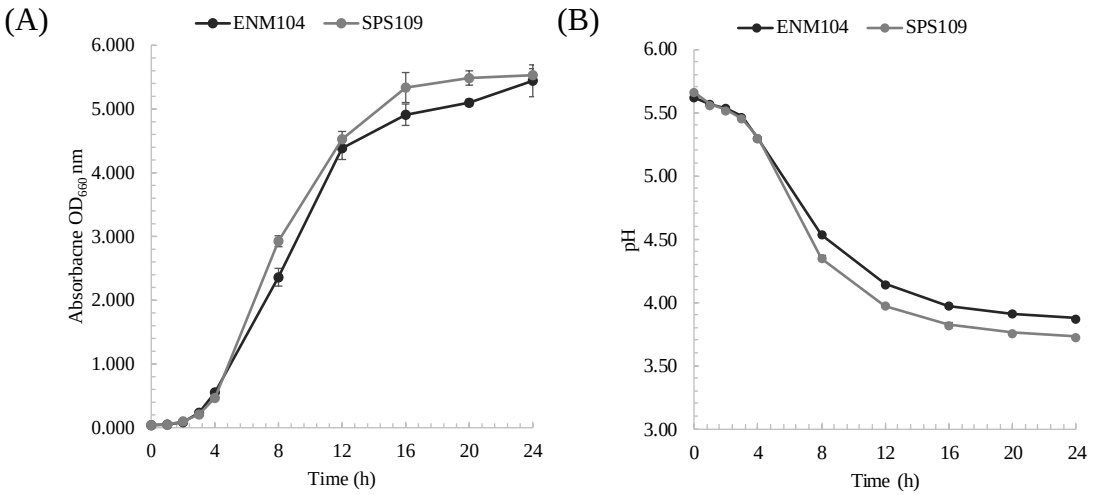


Figure 4-7. Kinetic curve of (A) bacterial growth and (B) pH of both contestant LABs including ENM104 (●) and SPS109 (●)

From kinetic growth, bacterial growth of both contestant LAB strains namely ENM104 and SPS109 was measured by time in h and absorbance in OD_{660} as shown in the Figure 4-7A that divided to three growth phase patterns including lag phase at the 0^{th} – 4^{th} h first, exponential phase at the 4^{th} – 16^{th} h and stationary phase at the 16^{th} – 24^{th}, while pH from bacterial growth was determined by time in h and pH value as shown in the Figure 4-7B. The growth pattern in pH depended on time was inversely similar to absorbance that also divided in three phases familiar with the above description. Moreover, pH from the bacterial fermentation was dropped with the lowest value in 24 h at 3.87 and 3.73 of ENM104 and SPS109, respectively. For the calculation of kinetic growth, Appendix D previews the calculation step. Results showed that *P. pentosaceus* ENM104 had specific growth rate at 0.8344 h^{-1} and generation time at 49.83 min, while *L. plantarum* SPS109 had specific growth rate at 0.7718 h^{-1} and generation time at 53.87 min. Moving to the comparison of cell numbers to cell turbidity, the results were shown in the Figure 4-8 and Table 4-11. Both regression equations composed of ENM104 with regression equation as $y = 0.6837x + 7.7644$; $R^2 = 0.9275$ and SPS109 with regression equation as $y = 0.6664x + 7.5885$; $R^2 = 0.9554$. The number of bacterial cells at 18 h in MRS broth medium was previewed as 9.19 and 8.93 log CFU/ml (Table 4-12), representing SPS109 and ENM104 strain, respectively.

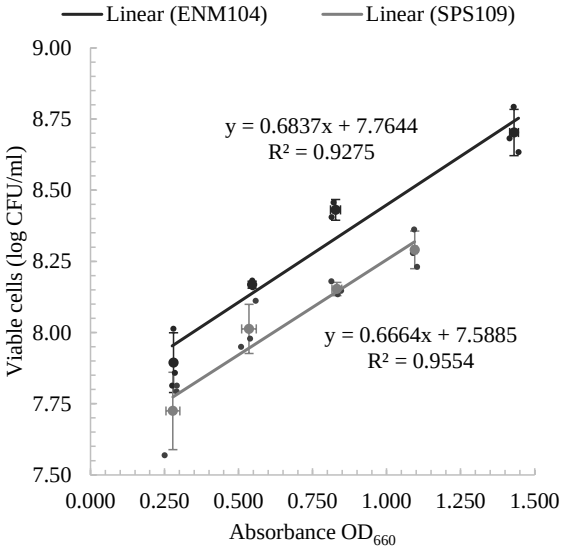


Figure 4-8. Relationship between viable cells (log CFU/ml) and turbidity in absorbance OD_{660} of both LAB strains including ENM104 with regression equation as y = 0.6837x + 7.7644; R^2 = 0.9275 and SPS109 with regression equation as y = 0.6664x + 7.5885; R^2 = 0.9554

Table 4-11. Composition between cell suspensions and diluents

Absorbance OD_{660}	**ENM104**			**SPS109**		
	undiluted OD_{660} = 1.844			undiluted OD_{660} = 1.833		
	Cell (µl)	**Diluent (µl)**	**Total (µl)**	**Cell (µl)**	**Diluent (µl)**	**Total (µl)**
0.250	150	3,000	3,150	200	3,000	3,200
0.500	350	3,000	3,350	450	3,000	3,450
0.750	625	3,000	3,625	850	3,000	3,850
1.000	1075	3,000	4,075	1400	3,000	4,400

Table 4-12. Cell numbers at 18 h incubation of potent LAB strains

LAB strain	**Cell number**	
	Numeral scale	**Logarithmic scale**
Lactobacillus plantarum SPS109	1.54×10^9	9.19
Pediococcus pentosaceus ENM104	8.46×10^8	8.93

7. Examination of probiotic properties of potent LAB strains

7.1. Hydrolysis of macromolecules

To start with the hydrolysis of macromolecules, Figure 4-9 and Table 4-13 give the results of both contestant strains. Two LAB strains (ENM104 and SPS109) were unable to hydrolyse starch as a carbohydrate source. However, only SPS109 was able to hydrolyse tributyrin as a lipid source. For protein hydrolysis, both strains were able to hydrolyse casein in skim milk agar medium, which was the component of milk, and SPS109 zone was larger than ENM104 strain.

7.2. Activity of bile salt hydrolases

Moving to bile salt hydrolase activity (Figure 4-10 and Table 4-14), both LAB strains produced bile salt hydrolase to lyse various bile salt derivatives including GCA, TCA and TDCA, excepted for GDCA. The concentration used at 5 mg/ml for each bile salt was appropriate for bacterial growth; meanwhile, the derivative named GDCA inhibited the bacterial growth.

7.3. Estimation of gastrointestinal fluid tolerance *in vitro*

In vitro probiotic property testing of both candidate strains named ENM104 and SPS109 was considered by Figure 4-11 at 10.18 ± 0.02 and 10.46 ± 0.04 log CFU/ml of ENM104 and SPS109 strains, respectively, which were the initial number of bacterial cells. After incubating in gastric fluid for 2 h, cells significantly decreased to 6.20 ± 0.12 and 7.39 ± 0.12 log CFU/ml of ENM104 and SPS109, respectively. After changing to intestinal fluid, the cell numbers were not significantly difference with previous fluid that confirmed no big change of cell numbers in the fluid transfer step. In the intestinal fluid condition, cells slightly increased and did not have any damage from intestinal fluid that contained bile salt and base condition. Finally, at the end of incubation at 6 h, it was found the number of the cells at 6.49 ± 0.00 and 7.63 ± 0.02 log CFU/ml for ENM104 and SPS109 strains, respectively.

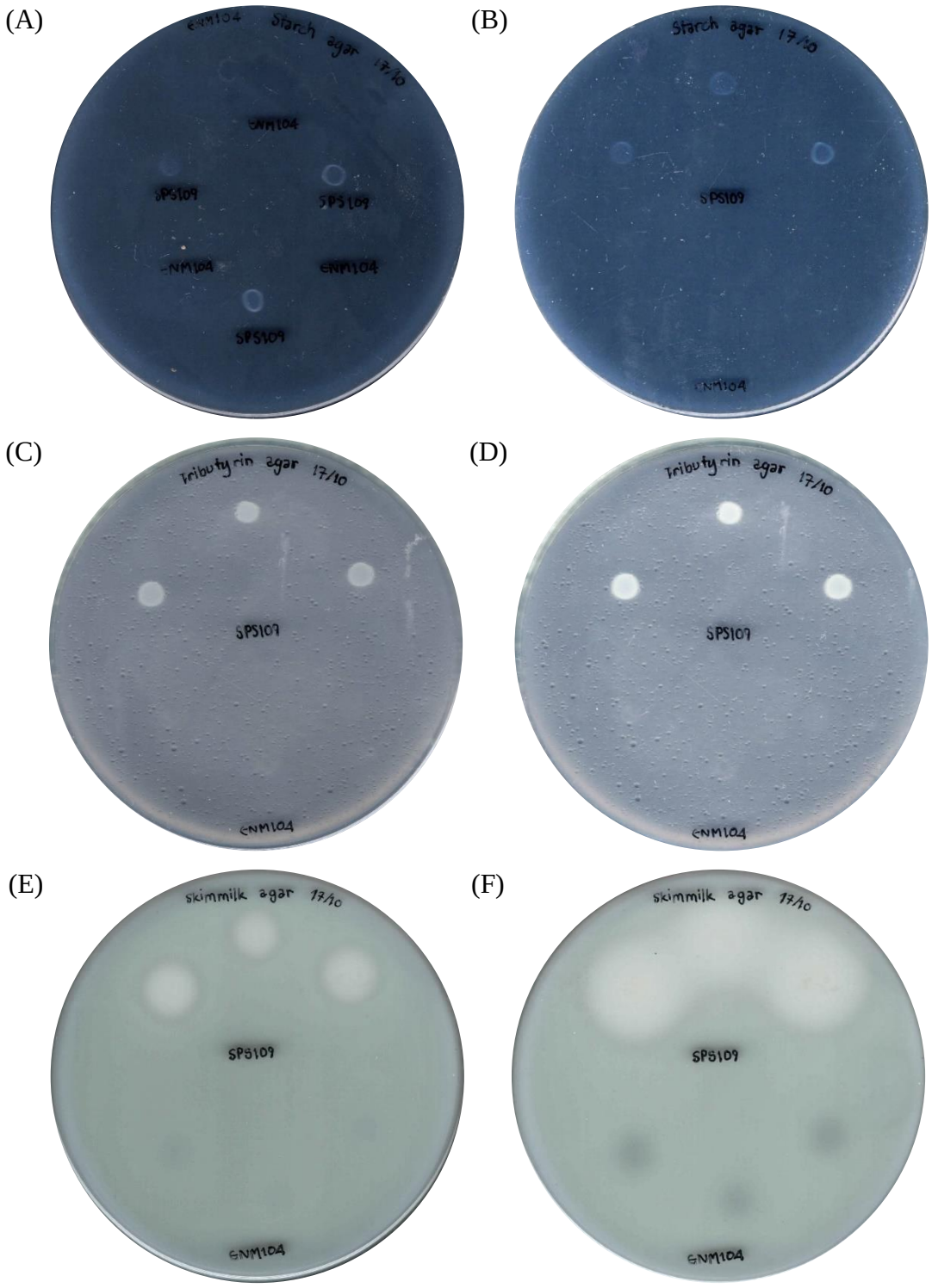


Figure 4-9. Hydrolysis activity of LAB strains including ENM104 and SPS109 with (A) 24-h-incubated starch agar medium, (B) 48-h-incubated starch agar medium, (C) 24-h-incubated tributyrin agar medium, (D) 48-h-incubated tributyrin agar medium, (E) 24-h-incubated skim milk agar medium and (F) 48-h-incubated skim milk agar medium

Table 4-13. Hydrolysis of macromolecules of LAB strains including *P. pentosaceus* ENM104 and *L. plantarum* SPS109

Medium test	**LAB strain**			
	ENM104		**SPS109**	
	24 h	48 h	24 h	48 h
Starch agar	-	-	-	-
Tributyrin agar	-	-	+	+
Skim milk agar	+	++	+	+++

Negative (-) refers as no opalescent zone appearance, meanwhile, positive (+, ++, +++) refers as tiny, moderate, huge zone appearance, respectively.

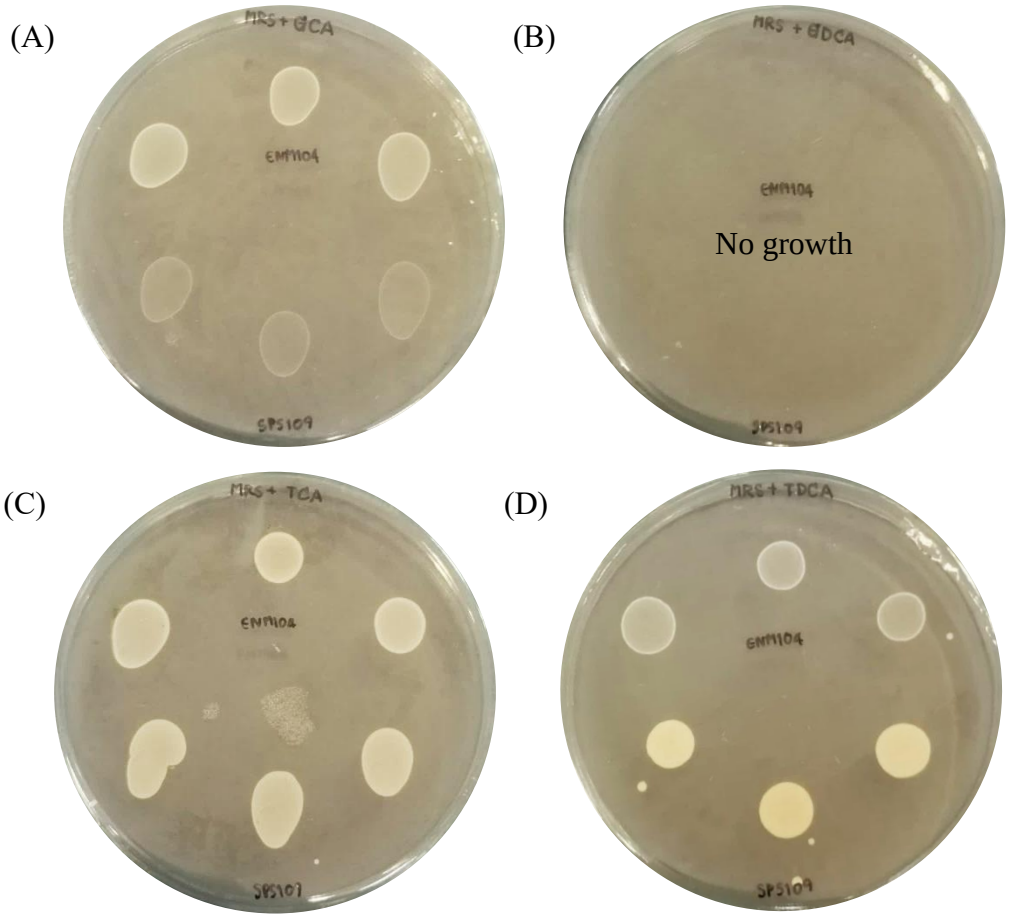


Figure 4-10. Bile salt hydrolase activity of both contestant LAB including ENM104 and SPS109 in various bile salt derivatises including (A) glycocholic acid; GCA, (B) glycodeoxycholic acid; GDCA, (C) taurocholic acid; TCA and (D) taurodeoxycholic acid; TDCA

Table 4-14. Bile salt hydrolase activity of LAB strains including *Pediococcus pentosaceus* ENM104 and *Lactobacillus plantarum* SPS109

Condition	**LAB strain**					
	ENM104			**SPS109**		
	24 h	48 h	72 h	24 h	48 h	72 h
Transparent zone						
GCA	+	+	+	+	+	+
GDCA	-	-	-	-	-	-
TCA	+	+	+	+	+	+
TDCA	+	+	+	+	+	+
Bacterial growth						
GCA	+	+	+	+	+	+
GDCA	-	-	-	-	-	-
TCA	+	+	++	+	+	+
TDCA	+	+	+	++	++	++

Negative (-) refers as no opalescent zone appearance, meanwhile, positive (+, ++, +++) refers as tiny, moderate, huge zone appearance, respectively.

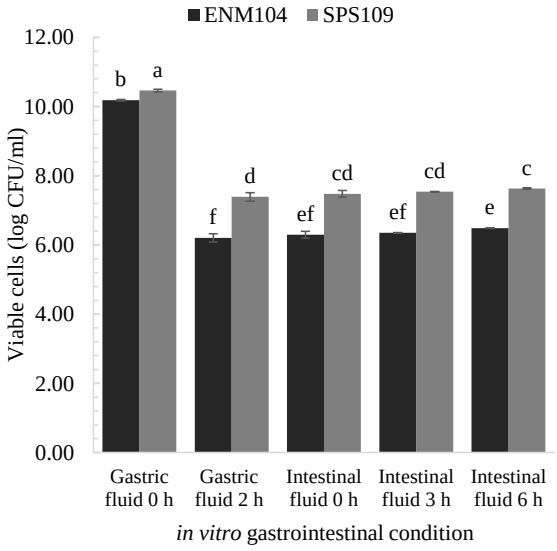


Figure 4-11. Viable cells of both contestant strains including ENM104 and SPS109 for probiotic properties *in vitro* test depending on time simulated by gastrointestinal tract with (■) as ENM104 and (■) as SPS109, different lowercase alphabets as significant differences ($p<0.05$) by *post-hoc* statistical analysis

7.4. Property of cholesterol-lowering activity

Turning to the property of cholesterol-lowering activity (Figure 4-12), the significant highest result of cholesterol reduction was observed in ENM104 strain at 14.88 ± 0.49 and 15.34 ± 1.12 percent for 48 and 72 h of incubation, respectively, with the minimum of bioaccumulation (Figure 4-2). For SPS109 strain, cholesterol-lowering content was 10.89 ± 1.06 and 10.05 ± 2.32 percent at 48 and 72 h, respectively, with no significant difference as well.

7.5. Cholesterol oxidase assay by ENM104 strain

Only ENM104 strain performed the enzyme activity determination due to the property of cholesterol-lowering activity in LAB basal medium supplemented with cholesterol. Table 4-15 gives the information about enzyme activity, protein content and specific enzyme activity of ENM104 at 72 h by cholesterol oxidase-resembled enzyme from crude extract of intracellular enzyme, representing 0.79 ± 0.18 mU, 0.09 ± 0.01 mg of protein content and 8.73 ± 2.00 mU/mg of protein, respectively; moreover, enzyme activity of this strain was risen with the highest at 72 h with significant differences ($p<0.05$), and specific enzyme activity was higher than 24 and 48 h that stayed in the same state of growth as stationary phase.

By the way, cholesterol oxidase was only detected by intracellular enzyme activity that no extracellular enzyme activity was observed (Figure 4-13B). Rosette-reddish colour was shown in 48 h and 72 h of intracellular enzyme activity assay of microtiter plates (Figure 4-13A).

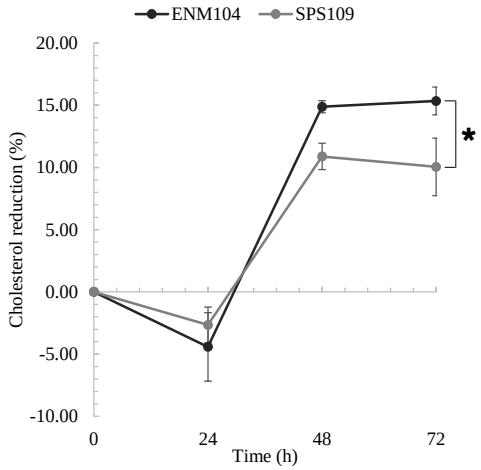


Figure 4-12. Property of cholesterol-lowering activity in MRS broth supplemented with 100 μg/ml cholesterol-PEG600 by (●) as ENM104 and (●) as SPS109 with (✱) as significant differences between both strains at 72 h of incubation ($p<0.05$)

Table 4-15. Intracellular cholesterol oxidase enzyme depending on times

Incubation Time (h)	**Intracellular cholesterol oxidase-resembled enzyme**		
	Enzyme activity (mU)	**Protein content (mg)**	**Specific enzyme activity (mU/mg)**
0	0.09 ± 0.01 [b]	0 ± 0 [c]	NA
24	0.09 ± 0.02 [b]	0.14 ± 0.01 [a]	0.67 ± 0.12 [c]
48	0.25 ± 0.09 [b]	0.13 ± 0.01 [a]	2.00 ± 0.73 [b]
72	0.79 ± 0.18 [a]	0.09 ± 0.01 [b]	8.73 ± 2.00 [a]

Different lowercase alphabets indicate significant differences ($p<0.05$) and NA means for not applicable.

(A) (B)

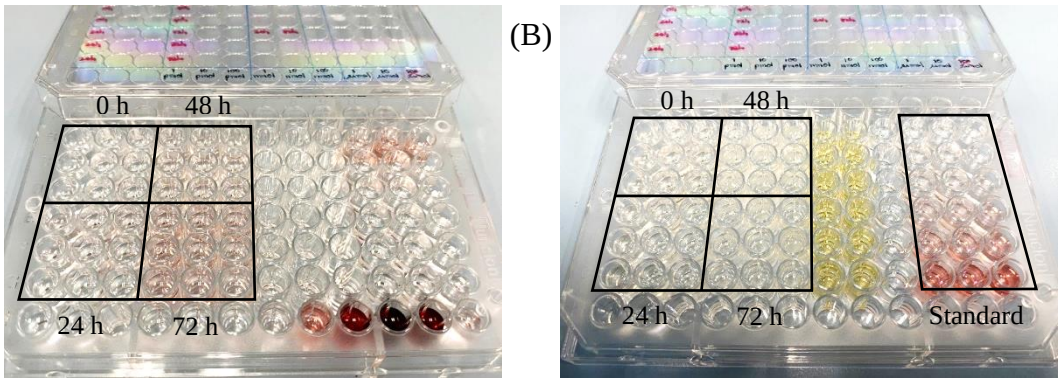


Figure 4-13. Microtiter plates of enzyme activity testing with (A) intracellular enzyme and (B) extracellular enzyme and standard substance, respectively

8. Tolerance to hydrogen peroxide

Tolerance of H_2O_2 (Figure 4-14) found that SPS109 strain could survive until 12 h in 1 mM H_2O_2 condition, but it was lower than ENM104 strain that could tolerate around 20 h.

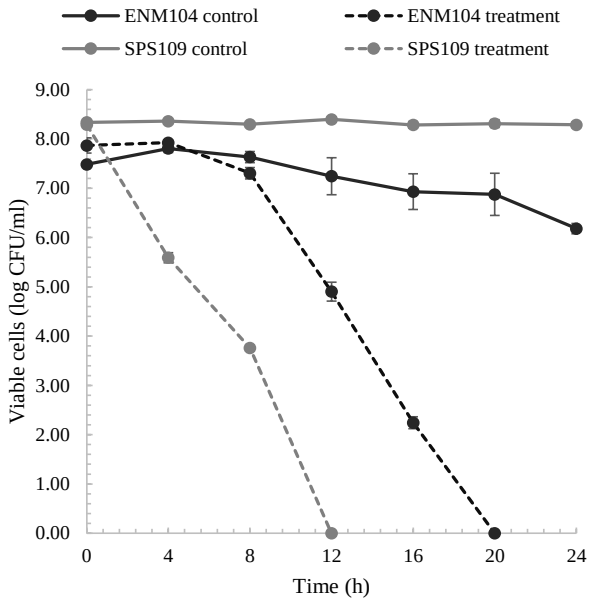


Figure 4-14. Viable cells of LAB strains including ENM104 and SPS109 for hydrogen peroxide tolerance with (—●—) ENM104 control, (--●--) ENM104 treatment, (—●—) SPS109 control and (--●--) SPS109 treatment

9. Determination of antioxidant activity and total phenolic content

9.1. Comparison of authentic compounds to determine antioxidant activity

Figure 4-15 illustrates the comparison results of various authentic compounds including trolox, L-ascorbic acid and gallic acid for antioxidant activity. To depict, DPPH was reported the information in % radical scavenging activity (% RSA) by authentic compound concentrations; meanwhile, ABTS was reported the information in % inhibition by authentic compound concentrations. For ORAC, the information was reported by net area under the curve (net AUC) by authentic compound concentrations as well. Trolox, L-ascorbic acid and gallic acid as authentic compounds produced IC_{50} of DPPH method at 304 ± 0 μM, 305 ± 5 μM and 118 ± 5 μM, respectively. Turning to IC_{50} of ABTS, it was displayed as 246 ± 2 μM, 257 ± 3 μM and 94 ± 1 μM by Trolox, L-ascorbic acid and gallic acid, respectively. For ORAC

result, IC_{50} cannot provide due to the difference of result report. To compare antioxidant activity of each authentic compound based on trolox, Figure 4-16 illustrates in term of mol TE/mol authentic compound.

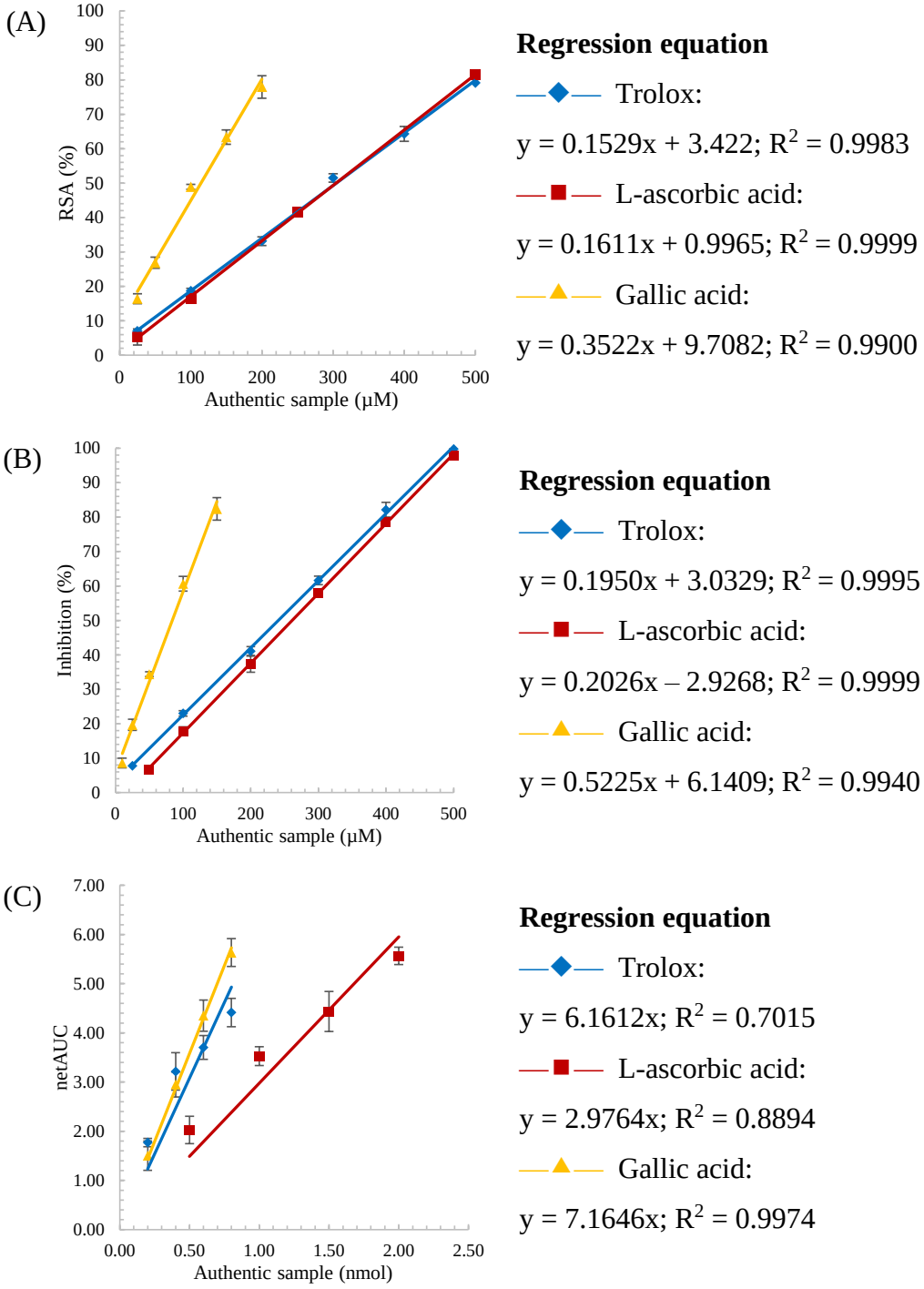


Figure 4-15. Comparison of regression line of trolox, L-ascorbic acid and gallic acid by (A) DPPH, (B) ABTS, and (C) ORAC

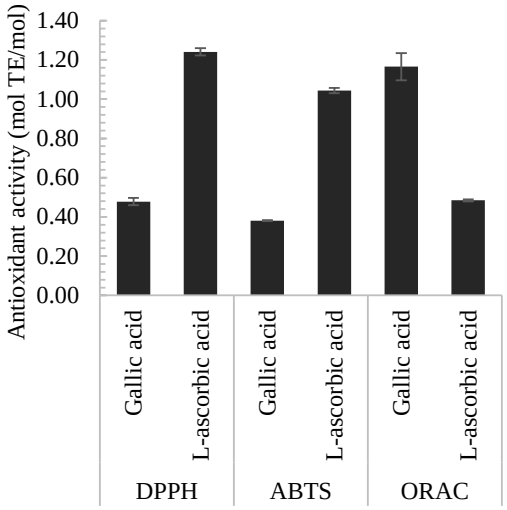


Figure 4-16. Antioxidant activity of each authentic sample comparison to Trolox by gallic acid and L-ascorbic acid

9.2. Antioxidant activity and TPC of selected LAB in MRS medium

For the estimation of antioxidant activity of both selected LAB strains including ENM104 and SPS109, the results were portrayed in the Figure 4-17. Antioxidant activity of culture supernatant was reported in term of µmole of trolox equivalent per ml of sample (µmol TE/ml); meantime, total phenolic content (TPC) was reported in term of µmole of gallic acid equivalent per ml of sample (µmol GE/ml). Antioxidant activity detected by ORAC method was illustrated with the highest result amongst with other methods with significant difference ($p<0.05$); however, DPPH and ABTS methods showed with no difference in term of statistics. For ORAC, the time at 48 and 72 h was previewed the highest antioxidant activity of both selected LAB strains, made up with 37.26 ± 0.77 and 34.01 ± 3.30 µmol TE/ml at 48 h of ENM104 and at 72 h of SPS109, respectively. On the other hand, antioxidant activity in DPPH and ABTS was shown the highest result at 24 h of fermentation of both selected LAB strains, representing 0.59 ± 0.00, 0.55 ± 0.01, 0.46 ± 0.01 and 0.44 ± 0.01 µmol TE/ml at DPPH of ENM104 and SPS109, and ABTS of ENM104 and SPS109, respectively. The amount of TPC was conformed with antioxidant activity and found that ENM104 and SPS109 showed the highest number of TPC at 24 h with 0.67 ± 0.00 and 0.68 ± 0.00 µmol GE/ml, respectively (Figure 4-17).

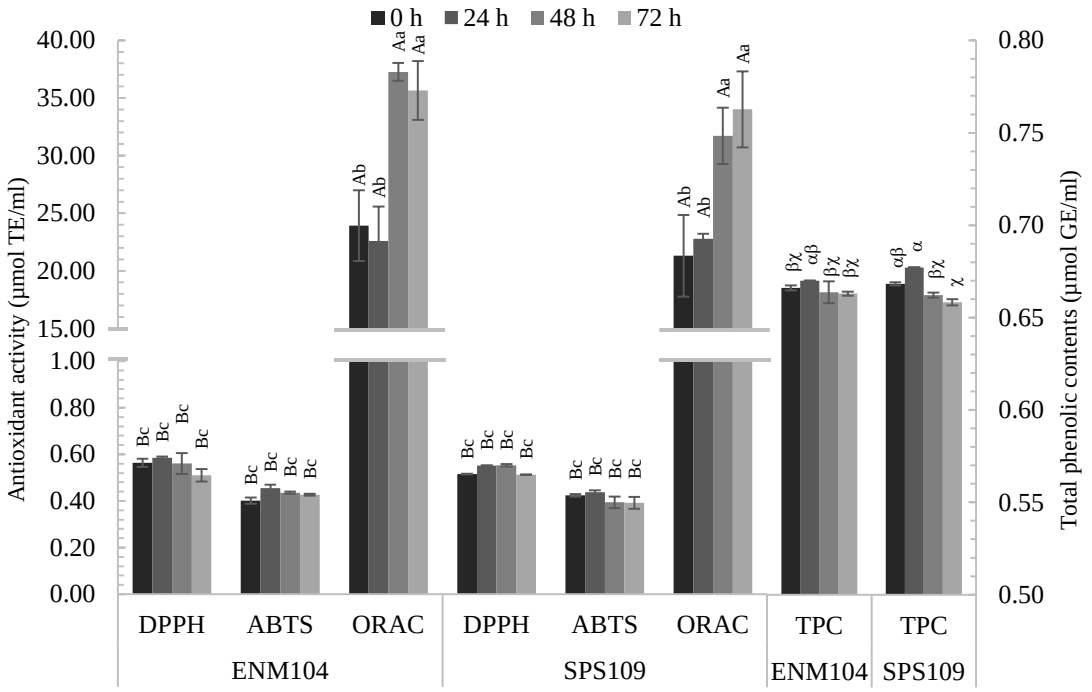


Figure 4-17. Antioxidant activity reported in µmol TE/ml of three detectable methods including DPPH, ABTS and ORAC with capital alphabets as significant differences amongst detectable methods and different lowercase alphabets as significant difference amongst all results ($p<0.05$), meanwhile, total phenolic content (TPC) reported in µmol GE/ml with Greek symbols as significant difference at $p<0.05$ of both selected LAB including ENM104 and SPS109 in MRS medium at 0 (■), 24 (■), 48 (■) and 72h (■)

10. Application of potent LAB strains as starter(s) for fermented dairy products

10.1. Fermented milk

Various parameters were detected for milk fermentation by both selected strains including ENM104 and SPS109 performing four sets of experimental designs composed of control set (uninoculated set), ENM104 set, SPS109 set and co-culture set. Figure 4-18 displays pH and acidity of these fermentations. pH of both culture combination was dropped to 5.32 ± 0.01 with significant difference ($p<0.05$) amongst other treatments while, it also generated the highest acid content at 0.35 ± 0.00 % with significant difference ($p<0.05$). Moving to Figure 4-19, viable cells were used to numerate the growth of both contestant strains. No growth was found in control set as uninoculated set, while the maximum growth was observed in SPS109 strain of single culture set and co-culture set, made up with 8.29 ± 0.01 and 8.27 ± 0.04 log CFU/ml with no significant difference between both sets. On the other hand, at 72 h of incubation sets of ENM104 as single and co-culture gave the maximum population at 7.41 ± 0.04 and 6.93 ± 0.01 log CFU/ml, respectively with significant difference ($p<0.05$) between both culture sets.

Moving to the next parameter, cholesterol-lowering activity of all treatment was illustrated in the Figure 4-20A. HPLC detection of cholesterol content in unfermented milk was 143.90 ± 4.30 µg/ml that was higher than the nutrition facts on the product description at 125 µg/ml for 15.12 percent (Table 2-7). Focusing on the range of 48 to 72 h incubation time that predicted for cholesterol oxidase production, cholesterol content was decreased from the initial cholesterol concentration by 15.71 ± 0.64 percent to 118.76 ± 0.91 µg/ml and 10.98 ± 3.80 percent in ENM104 single culture and co-culture sets, respectively compared with control set. Then, for this cholesterol-lowering level, ENM104 set previewed the result in significant difference ($p<0.05$) amongst other samples.

Focusing on efficiency of ACE inhibition, the highest ACE inhibitory activity was belonged to co-culture set at 72 h with significant difference ($p<0.05$) at 57.63 ± 2.97 percent inhibition (Figure 4-20B). All inoculated sets displayed the results of the efficiency after reaching at stationary phase around 24 h against control set that was stabilised the inhibition levels. Furthermore, SPS109 strain had a role to pulse for

higher activity than ENM104 set that showed the result at 56.02 ± 3.27 and 51.93 ± 4.54 percent inhibition at 72 h of incubation.

Turning to GABA and glutamic acid contents (Figure 4-21) and stochiometric calculation (Table 4-16), the average of initial glutamic acid content in milk was 43.39 ± 0.38 µg/ml. The trend of glutamic acid in ENM104 set showed a similar trend to control set, while the trend in SPS109 and co-culture sets reduced by 13.13 ± 3.86 and 16.02 ± 2.93 percent, from the initial concentration to 37.66 ± 1.31 and 36.44 ± 0.95 µg/ml at 72 h, respectively. The information at 72 h incubation of co-culture set was significant difference ($p<0.05$) amongst other treatments. Moving to GABA production, milk contained initial GABA content at 4.39 ± 0.01 µg/ml. From bacterial metabolism, the highest number of GABA production was found in SPS109 set within 72 h at 4.58 ± 0.00 µg/ml. To consider with stoichiometry, the highest production was SPS109 single culture set, representing 0.61 ± 0.05 percent. For loss contents, no mass of control and ENM104 set was indicated to loss from this reaction. Conversely, SPS109 single and co-culture sets showed the loss content from glutamic acid conversion to GABA with 12.53 ± 3.91 and 15.59 ± 2.97 percent, respectively, and both loss contents were significantly higher ($p<0.05$) than the other sets.

Moving to the results of antioxidant activity, numerous methods were used to detect antioxidant activity in fermented milk. To start with DPPH result (Figure 4-22A), the trend of ENM104 set sharply increased from 24 to 48 h of incubation time to touch the highest activity with significant difference ($p<0.05$) amongst other treatments, describing at 0.09 ± 0.00 µmol TE/ml of sample, while the trend of SPS109 and co-culture sets parallelly increased to the highest activity at 72 h. All bacterial-inoculated treatments illustrated that the activity started from the initial time of incubation until to the end of incubation. For ABTS result (Figure 4-22B), the highest result at 0.14 ± 0.02 µmol TE/ml of sample with significant difference ($p<0.05$) was belonged to ENM104 set at 24 h of incubation time; nevertheless, SPS109 set in the same incubation time illustrated at 0.10 ± 0.01 µmol TE/ml of sample. From the attempting of ENM104 strain in co-culture set, the trend of the activity at 24 h was risen to 0.12 ± 0.01 from 0.08 ± 0.00 µmol TE/ml of sample. For ORAC method result (Figure 4-22C), the highest activity at 2.58 ± 0.26 µmol TE/ml of sample with significant difference ($p<0.05$) was belonged to co-culture at 24 h of incubation time.

Both LAB strains supported for increasing of antioxidant activity by both single culture in similar time at 2.47 ± 0.12 and 2.40 ± 0.13 µmol TE/ml of sample by ENM104 and SPS109 sets, respectively. In all inoculated set after 24 h of incubation time, the activity declined especially ENM104 set that the result was nearly touched with control set. For the amount of phenolic compound by TPC detection (Figure 4-22D), the trend of TPC content in co-culture set was increased to the greatest content at 0.31 ± 0.03 µmol GE/ml of sample with significant difference ($p<0.05$) by supporting of SPS109 growth that substantiated by the single culture set of SPS109. However, the content in ENM104 set slightly enhanced and dropped down to near the control set.

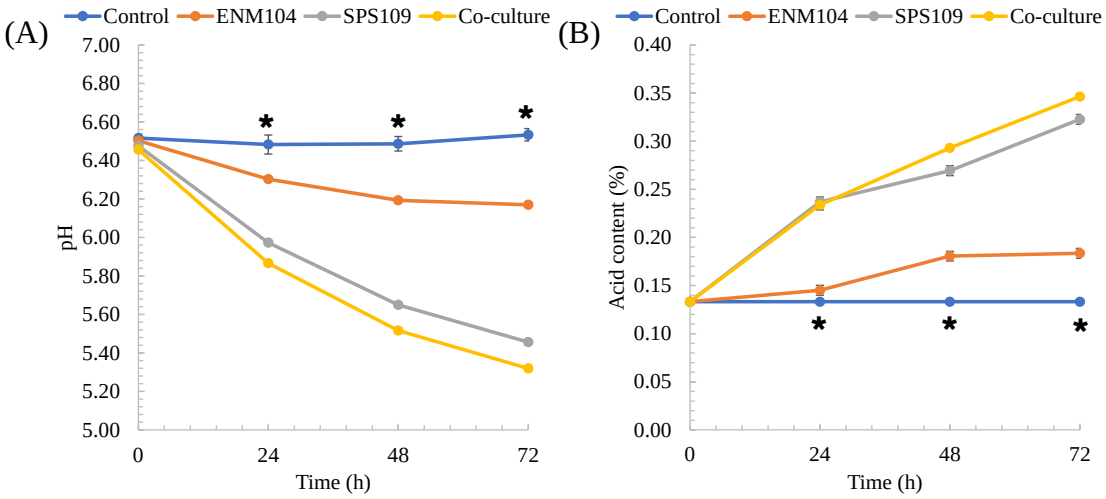


Figure 4-18. Milk fermentation for (A) pH and (B) acid content with legend description as (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture with asterisk (✱) representing significant difference ($p<0.05$) compared between control and the others sets

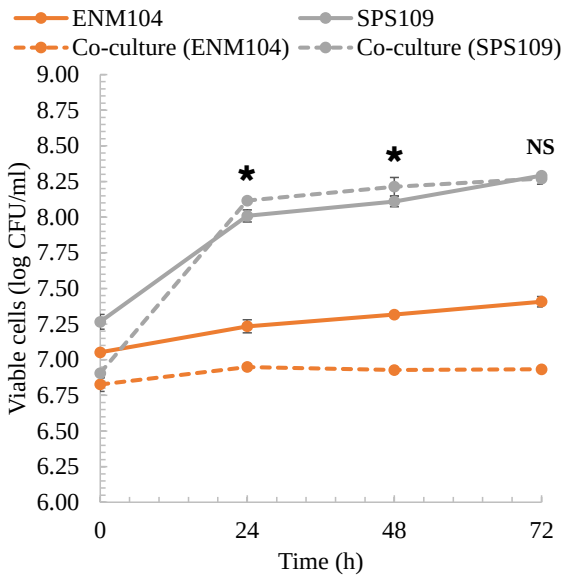


Figure 4-19. Viable cells of both selected LAB strains in milk fermentation of (—●—) ENM104 set, (—●—) SPS109 set, and co-culture set composed of (--●--) ENM104 and (--●--) SPS109, no appearance of viable cells in control set with asterisk (✱) showing for significant differences ($p<0.05$) and **NS** showing for no significant difference between SPS109 single and co-culture sets

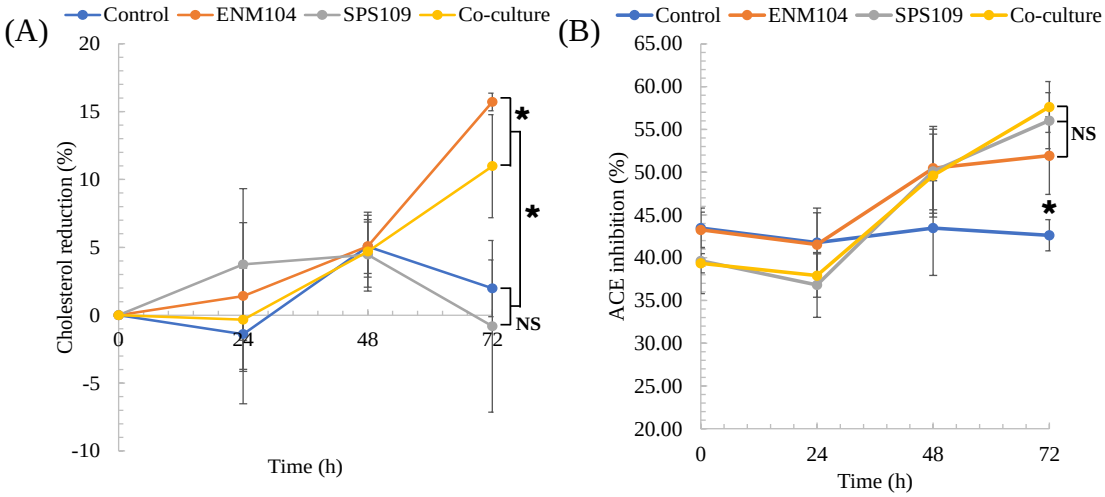


Figure 4-20. Milk fermentation with (A) cholesterol-lowering level with asterisk (✱) as significant differences ($p<0.05$) and **NS** as no significant difference on its pairing, and (B) ACE inhibitor efficiency with (✱) for significant differences compared with control and the other sets and **NS** depending on pairing, legend description as (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture

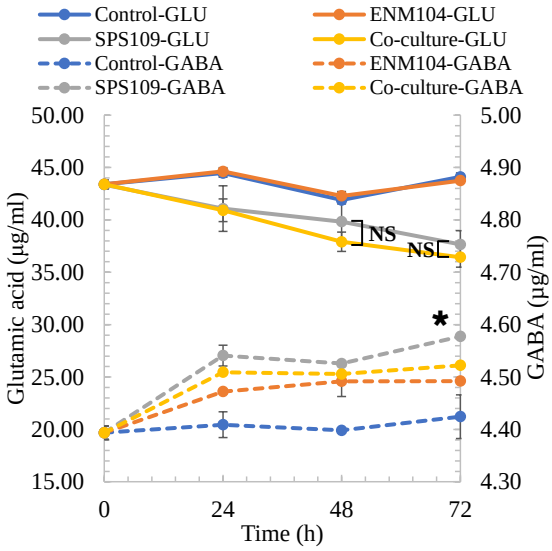


Figure 4-21. Milk fermentation for glutamic acid with legends including of (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture, and GABA content with legends including of (--●--) control, (--●--) ENM104, (--●--) SPS109 and (--●--) co-culture with asterisk (✱) as significant differences ($p<0.05$) between co-culture and the other sets and **NS** as no significant difference on their pairings

Table 4-16. The percentage of conversion efficiency of glutamic acid to GABA in milk fermentation

Treatment	**Time (h)**	**% Conversion efficiency**			
		Glutamic acid	**GABA**	**Total**	**Loss**
Control	0	100.00 ± 0.00 ^{ab}	0 ± 0.00 ^{e}	100.00 ± 0.00 ^{a}	0 ± 0.00 ^{c}
	24	102.48 ± 0.00 ^{ab}	0.05 ± 0.12 ^{de}	102.54 ± 0.12 ^{a}	-2.54 ± 0.12 ^{c}
	48	96.53 ± 0.03 ^{abc}	0.02 ± 0.03 ^{e}	96.56 ± 0.00 ^{ab}	3.44 ± 0.00 ^{bc}
	72	102.90 ± 1.80 ^{ab}	0.10 ± 0.09 ^{de}	101.73 ± 1.70 ^{a}	-1.73 ± 1.70 ^{c}
ENM104	0	100.00 ± 0.00 ^{ab}	0 ± 0.00 ^{e}	100.00 ± 0.00 ^{a}	0 ± 0.00 ^{c}
	24	102.84 ± 0.07 ^{a}	0.26 ± 0.04 ^{cd}	103.10 ± 0.11 ^{a}	-3.10 ± 0.11 ^{c}
	48	97.48 ± 1.81 ^{abc}	0.32 ± 0.05 ^{bc}	97.80 ± 1.76 ^{ab}	2.20 ± 1.76 ^{bc}
	72	100.83 ± 0.55 ^{ab}	0.32 ± 0.04 ^{bc}	101.15 ± 0.60 ^{a}	-1.15 ± 0.60 ^{c}
SPS109	0	100.00 ± 0.00 ^{ab}	0 ± 0.00 ^{e}	100.00 ± 0.00 ^{a}	0 ± 0.00 ^{c}
	24	94.74 ± 5.94 ^{abcd}	0.49 ± 0.02 ^{ab}	95.22 ± 5.92 ^{abc}	4.78 ± 5.92 ^{abc}
	48	91.89 ± 5.65 ^{bcd}	0.44 ± 0.06 ^{abc}	92.33 ± 5.71 ^{abc}	7.67 ± 5.71 ^{abc}
	72	86.87 ± 3.86 ^{cd}	0.61 ± 0.05 ^{a}	87.47 ± 3.91 ^{bc}	12.53 ± 3.91 ^{ab}
Co-culture	0	100.00 ± 0.00 ^{ab}	0 ± 0.00 ^{e}	100.00 ± 0.00 ^{a}	0 ± 0.00 ^{c}
	24	94.30 ± 3.34 ^{abcd}	0.38 ± 0.05 ^{bc}	94.38 ± 3.22 ^{abc}	5.32 ± 3.39 ^{abc}
	48	87.38 ± 2.90 ^{cd}	0.37 ± 0.04 ^{bc}	87.25 ± 2.72 ^{bc}	12.25 ± 2.95 ^{ab}
	72	83.98 ± 2.93 ^{d}	0.42 ± 0.05 ^{abc}	83.88 ± 2.95 ^{c}	15.59 ± 2.97 ^{a}

Lowercase alphabets refer to significant differences each column ($p<0.05$).

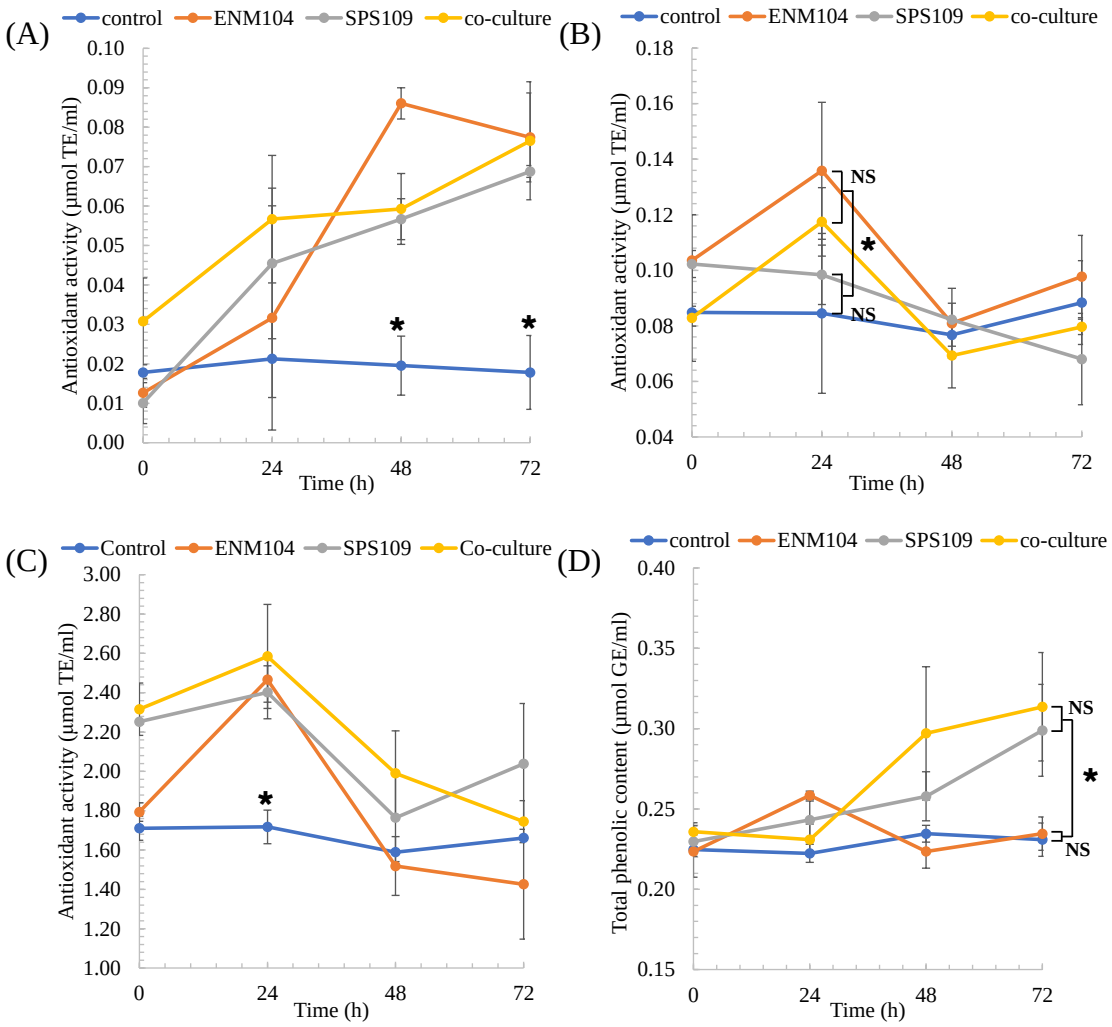


Figure 4-22. Milk fermentation results for antioxidant activity of both contestant LAB strains; (A) DPPH, (B) ABTS and (C) ORAC, and (D) TPC detection methods with legends including of (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture. Asterisk (✱) remains for significant differences ($p<0.05$) compared with control and other treatment sets for DPPH and ORAC method, but, in ABTS and TPC, asterisk and **NS** depends on their pairings.

10.2. Fermented whey

Figure 4-23 and Figure 4-24 illustrate changes of pH, acid content and viable cells in fermented whey using selected LAB strains including ENM104 and SPS109. Initial pH of whey fermentation was 5.46 ± 0.02 that was stable along fermentation time in control set. ENM104 set significantly decreased ($p<0.05$) from

initial pH to 5.02 ± 0.01. Moreover, SPS109 and co-culture sets also significantly decreased ($p<0.05$) pH at the maximum compared with control set, representing 4.34 ± 0.00 and 4.35 ± 0.01, respectively, but they were no significant difference between sets. The decrease of pH related to the increase of both acid content and viable cells. The maximum acid content belonged to SPS109 set at 0.51 ± 0.01 percent, and co-culture was closely to SPS109 set in 72 h at 0.50 ± 0.00 percent; however, ENM104 set showed 0.30 ± 0.01 percent of acid content. Turning to bacterial populations (Figure 4-24), the trend was familiar with acid content results. The highest viable cells with significant difference ($p<0.05$) compared with control set was observed in sets of SPS109 single culture and SPS109 in co-culture sets at 8.41 ± 0.02 and 8.37 ± 0.05 log CFU/ml at 72 h, respectively, but no significant difference was observed between these single and co-culture sets. On the other hand, ENM104 set displayed the result at 7.77 ± 0.06 log CFU/ml, while in the co-culture set, ENM104 strain showed the lowest growth at 7.19 ± 0.03 log CFU/ml.

For cholesterol determination in fermented whey (Figure 4-25A) found the cholesterol content of whey at 5.32 ± 0.15 µg/ml. To focus on the same periods for cholesterol reduction at 48 to 72 h of fermentation, single culture ENM104 and co-culture sets significantly decreased ($p<0.05$) cholesterol to 2.40 ± 0.72 and 3.32 ± 0.97 µg/ml, respectively. These decrease resulted for the great percentage of cholesterol reduction that was significantly higher ($p<0.05$) than control set but no significant difference between them, representing 54.86 ± 13.48 and 37.60 ± 18.28 percent for ENM104 single culture and co-culture sets.

Moving to ACE inhibition result (Figure 4-25B), a co-culture set performed the highest result at 72 h to 33.95 ± 4.95 percent inhibition with significant differences ($p<0.05$) from 25.70 ± 1.20 percent inhibition as the initial level of ACE inhibition result.

For the amounts of glutamic acid and GABA in fermented whey (Figure 4-26 and Table 4-17), the initial glutamic acid content found in fermented whey at 40.02 ± 0.08 µg/ml. With the conversion of glutamic acid to GABA, both trends of SPS109 and co-culture sets significantly declined ($p<0.05$) until 72 h but no significant difference between them, representing 19.36 ± 0.32 and 18.99 ± 0.14 µg/ml of glutamic acid, respectively. However, SPS109 set produced GABA to reach the peak ($p<0.05$)

at 24 h to 4.84 ± 0.01 µg/ml GABA or 4.74 ± 0.01 percent production from 3.51 ± 0.01 µg/ml of GABA which was the initial content of GABA in whey. The loss mass calculation displayed that the most loss content with significant difference ($p<0.05$) belonged to co-culture set at 72 h with 51.12 ± 0.13 percent.

Finally, antioxidant activity and total phenolic content results are illustrated in the Figure 4-27. The highest antioxidant activity based on DPPH found in ENM104 set at 72 h for 0.14 ± 0.00 µmol TE/ml of sample (Figure 4-27A). However, ABTS result, control set displayed the stable maximum result at 0.11 ± 0.01 µmol TE/ml of sample (Figure 4-27B). For ORAC result (Figure 4-27C), the trend of ENM104 and co-culture sets significantly enhanced ($p<0.05$) from the initial activity around 0.35 ± 0.09 µmol TE/ml of sample to the highest activity at 72 h for 0.69 ± 0.12 and 0.86 ± 0.22 µmol TE/ml of sample, respectively. Looking on the content of phenolic compounds (Figure 4-27D), co-culture set displayed the highest content at 72 h for 0.06 ± 0.01 µmol GE/ml of sample.

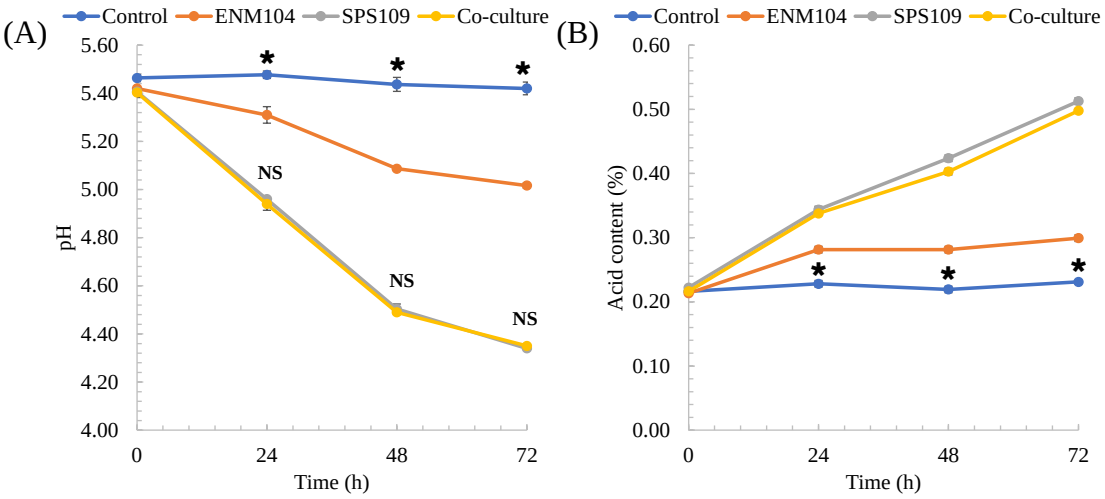


Figure 4-23. Whey fermentation for (A) pH and (B) acid content with legend description as (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture with asterisk (✱) as significant differences ($p<0.05$) compared with control and the other sets, and **NS** as no significant difference compared with SPS109 and co-culture sets

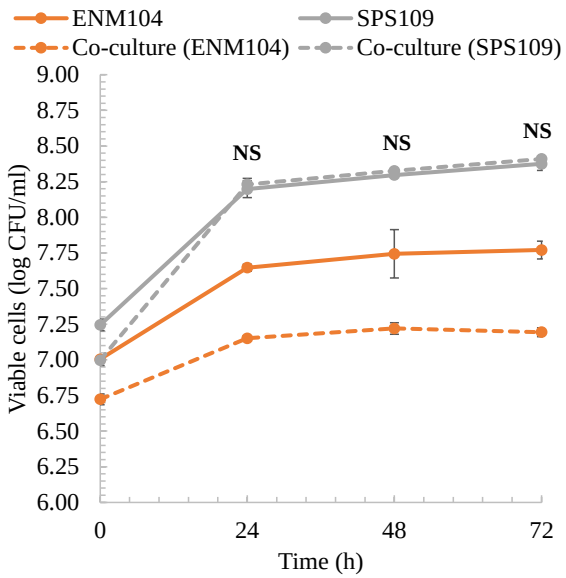


Figure 4-24. Viable cells of selected LAB strains in whey fermentation of (—●—) ENM104 set, (—●—) SPS109 set, and ENM104 count in co-culture set (--●--) and SPS109 count in co-culture (--●--) with **NS** as no significant difference between SPS109 single set and SPS109 strain in co-culture set.

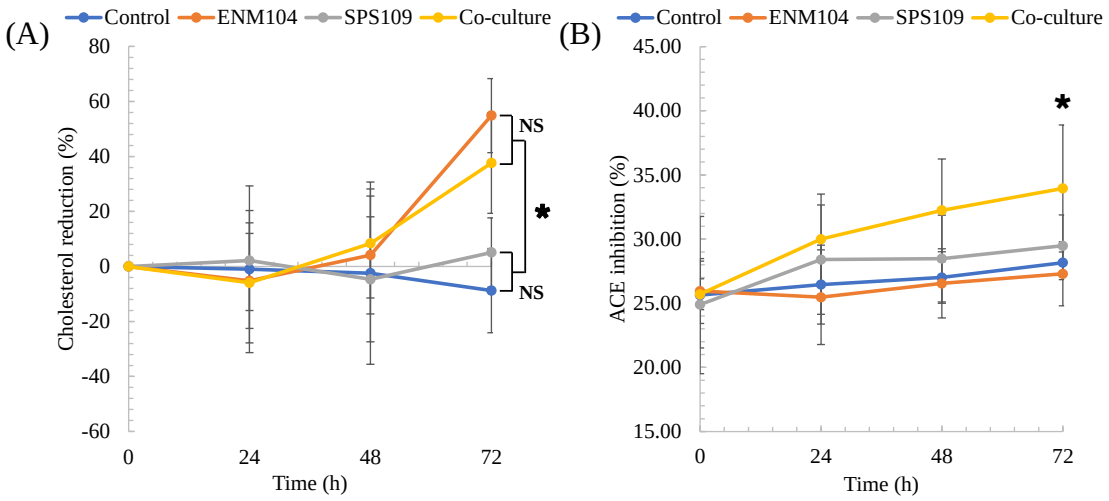


Figure 4-25. Whey fermentation with (A) cholesterol-lowering level, and (B) ACE inhibitor efficiency with legend description as (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture with asterisk (✱) in ACE inhibition as significant differences ($p<0.05$) compared with the other sets, while asterisk and **NS** as no significant difference describe for each pairing.

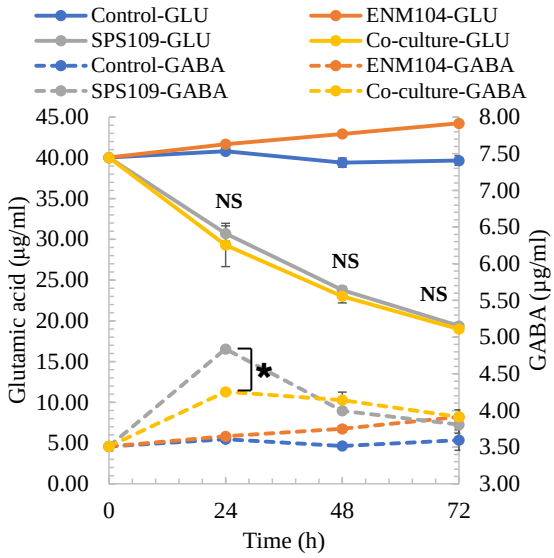


Figure 4-26. Whey fermentation for glutamic acid with legends including of (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture, and GABA content with legends including of (--●--) control, (--●--) ENM104, (--●--) SPS109 and (--●--) co-culture with asterisk (✱) as significant differences ($p<0.05$) on its pairing and **NS** as no significant difference compared with SPS109 and co-culture sets

Table 4-17. The percentage of conversion efficiency in the conversion of glutamic acid to GABA in fermented whey

Treatment	**Time (h)**	**% Conversion efficiency**			
		Glutamic acid	**GABA**	**Total**	**Loss**
Control	0	100.00 ± 0.00 ^{bc}	0 ± 0.00 ^{f}	100.00 ± 0.00 ^{c}	0 ± 0.00 ^{d}
	24	101.94 ± 0.17 ^{bc}	0.35 ± 0.12 ^{ef}	102.29 ± 0.29 ^{bc}	-2.29 ± 0.29 ^{de}
	48	98.47 ± 1.19 ^{c}	0.02 ± 0.10 ^{f}	98.50 ± 1.28 ^{c}	1.50 ± 1.28 ^{d}
	72	99.08 ± 1.67 ^{c}	0.30 ± 0.52 ^{ef}	99.39 ± 2.19 ^{c}	0.61 ± 2.19 ^{d}
ENM104	0	100.00 ± 0.00 ^{bc}	0 ± 0.00 ^{f}	100.00 ± 0.00 ^{c}	0 ± 0.00 ^{d}
	24	104.12 ± 0.48 ^{abc}	0.49 ± 0.11 ^{ef}	104.61 ± 0.59 ^{abc}	-4.61 ± 0.59 ^{def}
	48	107.26 ± 0.48 ^{ab}	0.85 ± 0.17 ^{def}	108.11 ± 0.65 ^{ab}	-8.11 ± 0.65 ^{ef}
	72	110.52 ± 0.37 ^{a}	1.43 ± 0.18 ^{cd}	111.95 ± 0.55 ^{a}	-11.95 ± 0.55 ^{f}
SPS109	0	100.00 ± 0.00 ^{bc}	0 ± 0.00 ^{f}	100.00 ± 0.00 ^{c}	0 ± 0.00 ^{d}
	24	76.73 ± 2.20 ^{d}	4.74 ± 0.01 ^{a}	81.47 ± 2.21 ^{d}	18.53 ± 2.21 ^{c}
	48	59.41 ± 1.14 ^{e}	1.73 ± 0.09 ^{cd}	61.15 ± 1.23 ^{e}	38.85 ± 1.23 ^{b}
	72	48.38 ± 0.69 ^{f}	1.06 ± 0.40 ^{de}	49.43 ± 1.09 ^{f}	50.57 ± 1.09 ^{a}
Co-culture	0	100.00 ± 0.00 ^{bc}	0 ± 0.00 ^{f}	100.00 ± 0.00 ^{c}	0 ± 0.00 ^{d}
	24	73.21 ± 6.48 ^{d}	2.66 ± 0.08 ^{b}	75.86 ± 6.56 ^{d}	24.14 ± 6.56 ^{c}
	48	57.51 ± 2.22 ^{e}	2.25 ± 0.41 ^{bc}	59.77 ± 2.63 ^{e}	40.23 ± 2.63 ^{b}
	72	47.44 ± 0.44 ^{f}	1.44 ± 0.32 ^{cd}	48.88 ± 0.13 ^{f}	51.12 ± 0.13 ^{a}

Lowercase alphabets refer to significant differences each column ($p<0.05$).

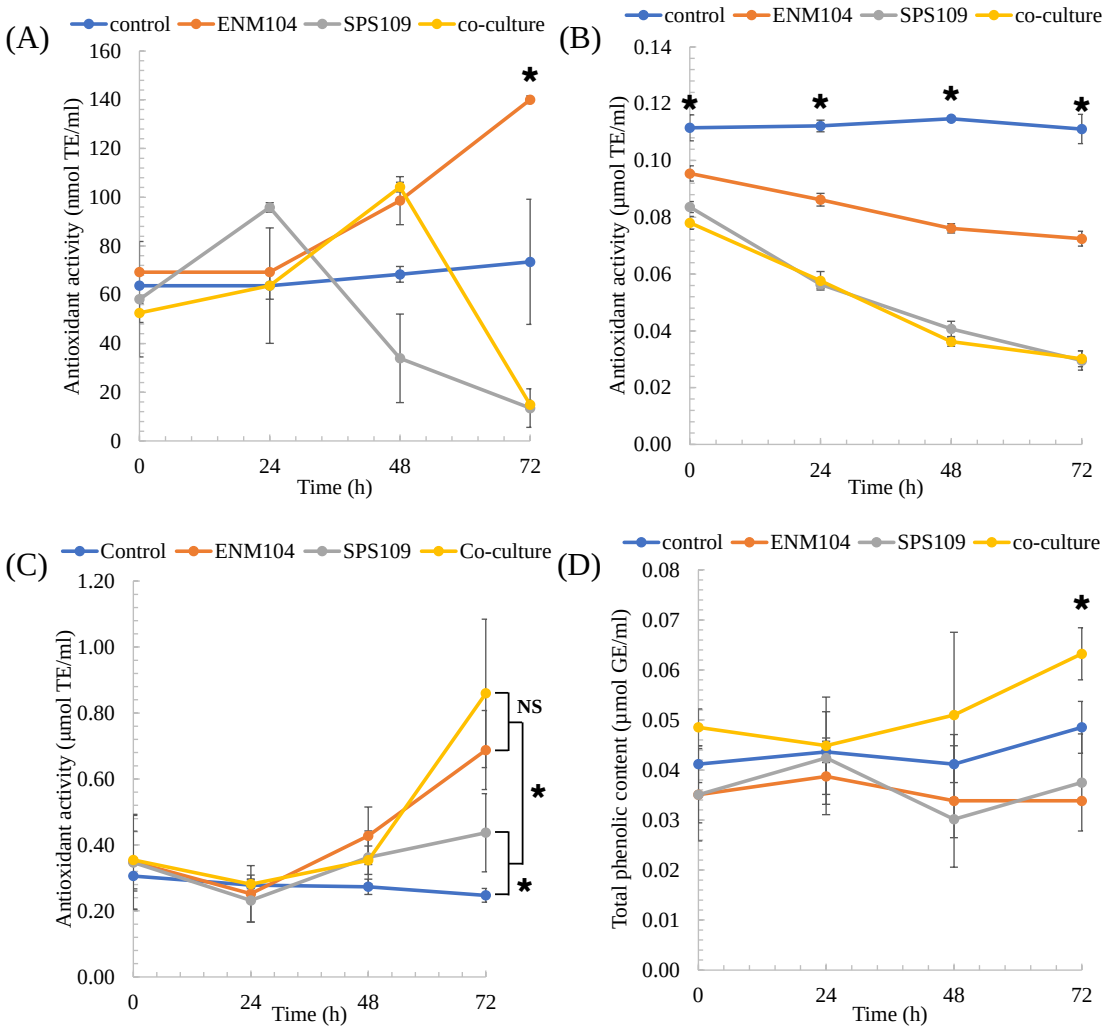


Figure 4-27. Whey fermentation for antioxidant activity of selected LAB strains was detected by (A) DPPH, (B) ABTS, (C) ORAC, and (D) total phenolic content with legends including of (—●—) control, (—●—) ENM104, (—●—) SPS109 and (—●—) co-culture. Asterisk (✱) represents for significant differences ($p<0.05$) compared between ENM104 set and the other sets in DPPH, control set and other sets in ABTS, co-culture set and the other sets in TPC, while, in ORAC method, asterisk and **NS** as no significant difference use to compared on their pairings.

CHAPTER 5

DISCUSSION

1. Isolation and selection of LAB

To isolate LAB from any samples usually uses pour plate technique from appropriate dilutions because LAB prefer microaerophilic condition. However, this present study streaking method was used as LAB are facultative anaerobe. This means that the isolated LAB should be easier to handle for application in case they might be probiotic candidates. Only 65 percent of collected samples (Table 4-1) were found LAB that conformed to 244 isolated LAB strains (Table 4-2). Feasibly, the collected samples contributing of the varieties including raw materials, fermented condition, fermentation types, and normal flora should be considered (Bovornreungroj 2018; von Wright and Axelsson 2012). Raw materials retain for the existence of normal flora depending on ecosystem and diversity, particularly LAB. For example, sea fish, sea shrimp and sea molluscs contain LAB strains which prefer saline condition to grow; moreover, the use of these sea creatures to produce fermented foods adds salt for taste satisfaction. The outcome from the fermentation is the high osmolarity to affect for the selection of LAB strains that only halotolerant LAB strains can grow. With the isolation method, MRS medium without any salt for supplementation could not isolate those LAB strains out from the samples because the application used for fermented dairy products does not require for salt addition. Then non-halotolerant LAB may not be found in those fermented foods including Bu-du, Ka-pi (shrimp paste), Hoi-sieb and Jing-jang which contained salt in range of 14.0 – 40.1 percent in shrimp paste and 19.4 – 20.6 percent in Bu-du. Typical LAB discovered in samples were *Tetragenococcus halophilus* and *T. muriaticus* (Tanasupawat and Visessanguan 2014). However, the sampling of those fermented foods was planned due to the expectation of cholesterol containing in the sea creatures and the possibility of LAB finding with no requirement salt to grow. To be concluded, the isolated LAB strains were led to assay in advance with primary screening for inspection of cholesterol-lowering properties.

2. Inspection of cholesterol-lowering LAB

A total of 139 isolated LAB or 56.97 percent was endured in LAB basal medium supplemented with 100 µg/ml cholesterol as primary screening for cholesterol survival property (Table 4-3). This medium contained essential minerals to support life-prolonging especially 0.1% (w/v) yeast extract that was enough for cell survival. Moreover, yeast extract might support more carbon source than only cholesterol supplementation. Then the next experiment, basal medium supplemented with cholesterol without yeast extract was used to screen LAB. Ten isolates or 7.19 percent in secondary screening survived in testing medium with only cholesterol as a carbon source (Table 4-4). Possibly, isolated LAB could prolong and tolerate their life in the medium containing cholesterol as carbon source. Five LAB strains were selected in tertiary screening based on their higher cell proliferation; however, only three strains including ENM104, NNM205 and NNM304 showed the significantly higher of viable cells in cholesterol-containing medium than in control as non-cholesterol supplementation (Figure 4-1). Feasibly, they were able to grow using cholesterol as a carbon source with the basis of nutrient limitation to allow only carbon source containing in the medium to survive. However, for utilisation of cholesterol, five strains were investigated in the next experiment to check the cholesterol level with analytical method to obtain the best contestant to reduce cholesterol in the designed medium.

3. Use of cholesterol by potent LAB

Concept of this experiment aimed to investigate the best cholesterol loss content from bacterial fermentation by the calculation from remaining cholesterol content in culture supernatant and removing of cholesterol contents into their cells called bioaccumulation. From the previewed results in the Table 4-5 and Figure 4-2, ENM104 strain displayed the best result for the loss of cholesterol content in culture supernatant and total cholesterol loss content. The loss of cholesterol content was the most interesting owning to the possibility of bacterial cells to use cholesterol as a carbon source to survive and grow. Familiar publication to screen the best cholesterol decomposition reported that bacterial isolation from both fermented dairy products and dairy products in basal medium supplemented with cholesterol found many strains of

cholesterol-lowering bacteria which can also utilise cholesterol for cell survive with the maximum at 75.30 percent in *Enterococcus hirae* Mil-31, but this bacterium is not LAB (Yehia et al. 2015). To be concluded, ENM104 strain was selected for the next experiment with the potential cholesterol-lowering activity by investigating cholesterol oxidase that degrades the cholesterol.

4. Investigation of γ-aminobutyric acid-producing LAB

Ten isolated LAB strains from secondary screening were collected to produce γ-aminobutyric acid (GABA) by screening with TLC (Figure 4-3) that only SPS109 showed for the ability to release GABA. Then, this strain was collected for evaluation the amount of GABA by HPLC. As there was only SPS109 strain that had the activity to change glutamic acid to GABA by glutamic acid decarboxylase (GAD) enzyme (Figure 2-10). In the fermented process of SPS109, glutamic acid was increasingly transferred by GAD enzyme to GABA until fermentation time stop at 72 h. With the information from the percentage of conversion efficiency (Table 4-6), MSG was decreased around one-third folds to transform to GABA that generated from that one-third folds within 72 h of incubation. This generation referred to 1,157.01 ± 4.76 µg/ml of GABA concentration. To tightly consider, loss from this conversion was tiny appeared in the fermentation process from both mole of GABA and glutamic acid combination because, in the reaction as Figure 2-10, carbon dioxide was generated as by-product of enzyme activity to lose in the atmosphere. Nevertheless, possibly form this study, GABA significantly increased along the incubation time. To compare with other reports, GABA production from LAB was popular for functional food production. *Pediococcus pentosaceus* HN8 converted from glutamic acid to GABA at 9,060 µg/ml in 24 h from MRS broth medium supplemented with 5 mg/ml MSG (Ratanaburee et al. 2013b). Another example to preview as *Weissella hellenica* SB105 produced GABA from MRS broth medium supplemented with 10 mg/ml MSG in 96 h at 7,690 ± 210 µg/ml (Barla et al. 2016). For the GABA production of SPS109 strain, it was a much lower than the referred reports. This might be efficiency of GABA conversion enzyme of each strain, initial MSG concentration and incubating condition.

5. Identification of selected LAB strains

According to morphological characteristics (Table 4-7) provide useful information for bacterial DNA extraction and tracking of bacteria when performed co-culture experiment. All strains of LAB identification results received from the genomic identification tool; however, the phenotypic information including 49 types of carbohydrate fermentation for biochemical properties (Table 4-8) was also used for identification, but it was low accuracy for some LAB. Based on the biochemical identification, sometimes the results were not similar to the most similarity percent, so it could be used other information to consider. For example, as an obvious case, LAB strains namely NNM205 and NNM304 (Table 4-9) displayed the highest of percent similarity that was lower than 50 percent, but the most similarity showed as *Lactobacillus brevis* that morphology characteristics including cell shape and arrangement differed from those strains themselves. Both strains were tetrads cell shape characteristics that showed true accuracy as *Pediococcus pentosaceus* in the third rank with 10 percent similarity.

On the other hand, genomic identification (Table 4-10) presented the most similarity as *P. pentosaceus* that was proper with cell characteristic described in the above. Thus, nowadays, molecular identification considering by taxonomical relationship with a phylogenetic tree in the Figure 4-6 has been admitted as the most effective tool for bacterial identification. Around 1,500 bp length referred to 16S rRNA (Figure 4-5) were used as the primary molecular tool to distinguish the difference amongst the bacteria that 97 percent are a cut out criterion for new species (Janda and Abbott 2007). Based on a phylogenetic tree (Figure 4-6), two groups including SPS109 and the other five strains were divided as the basis of evolutionary distance, but SPS109 strain still had the remote relationship with the other five strains. This remote relationship related to some conserved DNA fragment which convergently developed to be LAB.

With the obvious information of biochemical and molecular tests, both methods can allow to use as a tool for identification of LAB strains. The benefit of biochemical test is easy to practice without special technique knowledge. Moreover, the information of sugar fermentation can apply to use in some experiments like modified MRS medium described in advance topic below.

To be concluded, six LAB strains can be divided in two groups with the genus name in the genotypic identification with the obvious separation in the phylogenetic tree. Five LAB strains isolated from Thai fermented pork or Nham including ENM104, ENM203 and ENM204 from Mukdahan province in north-eastern region of Thailand, and NNM205 and ENM304 from Chiangmai province in northern region of Thailand were *P. pentosaceus*. This genus of LAB is typically found in Nham as the normal flora in ground pork as main raw material. It has a role in the first three days of fermentation by utilisation of glucose to generate lactic acid as the activity of homofermentative LAB. As pH was down to around 4.5 indicating the growth of other LAB such as *L. plantarum* (Ratanaburee et al. 2013b). This supports that five strains of *P. pentosaceus* might be isolated from around the first three days fermentation. Another strain namely *L. plantarum* SPS109 was isolated from Pla-som as a fermented fish from in Narathiwat province in southern of Thailand. This genus is typically found in Pla-som as normal flora of *Puntius gonionotus* (Ta-pian fish) (Tanasupawat and Visessanguan 2014). In this study, most selected LAB strains were isolated from various samples that are salt-based fermented foods, and none from dairy products. Thus, this is the main reason that why this research found many strains in the genus of *Pediococcus* spp. as this genus can tolerate to low salt (von Wright and Axelsson 2012).

6. Kinetic growth of both selected LAB strains

Two strains after finishing the identification step were selected as the contestant strains for further experiments based on their activities including cholesterol-lowering activity represented by *P. pentosaceus* ENM104 and GABA-producing activity made up with *L. plantarum* SPS109. Then these two strains were used as starter culture(s), and, to know the insight growth characteristics, the study of kinetic experiment is mandatory to perform. Based on specific growth rate and generation time of ENM104 and SPS109 strains at 0.8344 and 0.7718 h^{-1}, and at 49.83 and 53.87 min, respectively (Appendix D), these data are important to be used in further experiments including fermentation time for an example. Figure 4-7 portrays that after 16 h of incubation time, bacterial cell reached to stationary phase, and this is exceptional information to use for preparing inoculums. Cell numbers by viable cells at 18 h incubation (Figure 4-8) was used to estimate the initial cell number as inoculums

including the probiotic experiment. While 18 h of incubation was the suitable time to collect cell for inoculum preparation i.e. the viable cells, approximately cell numbers of both strains were 9 log CFU/ml.

7. Examination of probiotic properties of potent LAB strains

7.1. Hydrolysis of macromolecules

To consider for probiotic properties, thereby, more benefits of both selected LAB on their digestion of macromolecules were investigated. Figure 4-9 and Table 4-13 show that both of them no amylase activity; however, SPS109 strain was able to digest lipid in form of tributyrin. In contrast, both strains digested casein. This is not unsurprised that both were isolated from fermented pork and fish that enriched with protein and lipid. Protein hydrolysis was mostly important for study of angiotensin-converting enzyme (ACE) inhibition in fermented milk related for reducing the blood pressure (Georgalaki et al. 2017). Possibly, with proteolytic activity, our selected LAB might hydrolyse casein to specify for inhibition of ACE. This was useful for further experiment in fermented milk application.

7.2. Activity of bile salt hydrolases

As probiotic LAB strains, they must survive in digestive system until reach to colon of host. Hence, their tolerance on bile salts were examined on the basis of bile salt hydrolase activity (Figure 4-10 and Table 4-14). Both selected LAB hydrolysed almost of bile salts tested, excepted GDCA. Possibly, GDCA might have higher concentration to inhibit bacterial growth. With other publication (Ratanaburee et al. 2013b), *P. pentosaceus* HN8, NH102 and NH116 had the result in term of positive aspect for bile salt hydrolase of all bile salts tested. However, *P. pentosaceus* ENM104 yielded the result in different term indicating that difference strains can have different properties. Another publication (Cebeci and Gürakan 2003), other *L. plantarum* strains were tested with four types of bile salts as above in MRS agar that no growth appearance in GCA and TDCA supplementation. Feasibly, the result depended on tested strains.

It is important to test bile salts hydrolase of LAB strains for probiotic properties, so, for the *in vitro* gastrointestinal (GI) fluid tolerance, these compounds are one of the ingredients to challenge the bacterial survival with mimicking of GI tract.

7.3. Estimation of gastrointestinal fluid tolerance *in vitro*

In vitro probiotic property testing for biological barrier (Figure 4-11) in MRS medium was used as food candidate which was the most simplify to test. In the normal situation of the GI tract, bacterial cells were enrolled to the GI tract with some diets that could protect hazardous condition of the digestive tract and prolonged cells to survive. Population of *P. pentosaceus* HN8 reduced the number from 8.98 to 8.84 log CFU/ml in gastric fluid (Ratanaburee et al. 2013b). Moreover, when finishing incubation of intestinal fluid, cells remained around 5.30 log CFU/ml that was lower than the number in this experiment at 6.49 ± 0.00 and 7.63 ± 0.02 log CFU/ml for ENM104 and SPS109 strains, respectively, with initial cells roughly 10 log CFU/ml.

The necessary information to select probiotic strain was reported in the meeting at Canada to define the probiotic criteria (Morelli and Capurso 2012). *In vitro* test was one of the various suggestion tests that contained of gastric fluid tolerance, bile salt resistance, mucus adhesion, bile salt hydrolase activity, antimicrobial activity of pathogenic bacteria, and reduction of pathogenic bacteria to adhere to the intestinal wall. Besides, to confirm as a potential probiotic strain, *in vivo* test should be performed in animal and individuals. Then it was concluded that this experiment was the fundamental test leading to the candidate probiotic bacteria.

7.4. Property of cholesterol-lowering activity

For the property of cholesterol-lowering activity in MRS broth (Figure 4-12), around 15 percent of cholesterol lowering content was found in ENM104 strain that was higher than % cholesterol-lowering content in LAB basal medium supplemented with cholesterol. Possibly, the increase of cholesterol-lowering content in MRS medium might be from the increase of cell numbers. With the massive cell numbers, cholesterol oxidase enzyme might increase; however, the choice related with cholesterol accumulation in the cells were not cut out, but in this experiment did not

measure the number of cholesterol contents in the cells. Then it was not substantiated that how much of cholesterol content was stored in the cells. For SPS109, it was not curious that this strain also reduced cholesterol in cell-free supernatant as ENM104 strain. SPS109 strain was passed step of selection with similar as ENM104 for cholesterol-lowering property. Feasibly, cholesterol content in this culture might be assimilated in the cells and degraded with enzyme as ENM104 that was come from cholesterol oxidase enzyme. Furthermore, this discussion might be predictable information. To compare with other publication (Ratanaburee et al. 2013b), *P. pentosaceus* NH116 reduced the cholesterol content in cell-free supernatant down to 43.32 ± 1.01 percent that was a huge difference. Probably, it depended on strain type and difference of cholesterol type in the experiment. 70 µg/ml of cholesterol in ethanol was used in this publication, while 100 ug/ml of cholesterol-PEG600 was supplied for this present study.

Compared with the other publications, the cholesterol-lowering activity has been performed in MRS medium supplemented with cholesterol with several examples to support. The loss of cholesterol increased with the cell numbers after longer incubation than 24 h around 40 percent cholesterol loss with cells increased to around 9 log CFU/ml. This showed that the cholesterol loss depended on cell numbers that used other carbon source in MRS for growing and could be possible that the loss of cholesterol occurred by bioaccumulation (Pereira and Gibson 2002; Ratanaburee et al. 2013b; Tomaro-Duchesneau et al. 2014).

7.5. Cholesterol oxidase assay by ENM104 strain

Table 4-15 shows increasing of cholesterol oxidase activity along with longer incubation for 72 h. The activity period started in stationary phase of bacterial growth. For protein content, after 24 h of incubation, protein content was stable and tiny declined after 48 h of incubation. This indicates that cholesterol oxidase is an inducible enzyme which took times for cells to produce and establishes after ending of exponential phase that cells used glucose to be empty.

When consider altogether with cholesterol-lowering properties in MRS broth medium supplemented with 100 µg/ml cholesterol-PEG600, cholesterol was decreased since 24 h that was shown 48 h of incubation result at 14.88 ± 0.49 percent

and 72 h result at 15.34 ± 1.12 percent of cholesterol-lowering activity (Figure 4-11). This indicates that the loss might be from bioaccumulation as testing of cholesterol oxidase. Normally, probiotic properties with cholesterol-lowering property was usually described with various directions (Tsai et al. 2014) including removing and storing of cholesterol to inside the cells, attaching cholesterol molecule with some non-polarity molecule at cell wall, some chemical production from LAB including ferulic acid to prohibit hepatic HMG-CoA reductase with promotion of acidic sterol excretion to faeces (Tomaro-Duchesneau et al. 2014) and production of short-chain fatty acids between cell growth, conversion of cholesterol to non-absorbable molecule like coprostanol, and deconjugation of bile salts by bile salt hydrolase (Figure 2-9) which were derivatised molecules of cholesterol to destruct the property of re-absorption of cholesterol derivatives back to circulatory system.

For the activity of cholesterol oxidase enzyme (Allain et al. 1974), this enzyme changes cholesterol by acting with hydroxyl group for conversion to carbonyl group by elimination of a hydrogen atom out from the molecule. Metabolite generates from this enzyme is called 4-cholesten-3-one, however, cholesterol is further to generate 5-cholesten-3-one that was performed as instable state, and this molecule is switch electron to the next position that is shown as 4-cholesten-3-one as stable molecule. This molecule is shown in the initial state of cholesterol conversion cassette by which numerous enzymes need to completely degradation. Water molecules will temporally hold up that hydrogen atom to make a hydrogen peroxide molecule. This hydrogen peroxide is detected by enzyme conversion named horseradish peroxidase to coloured molecules with the helping of 4-aminoantipyrine and phenol molecules to quinonimine dye showing in rosette-red colour detected with 500 nm spectrophotometry. To more understanding, Figure 5-1 previews how the reaction was happened.

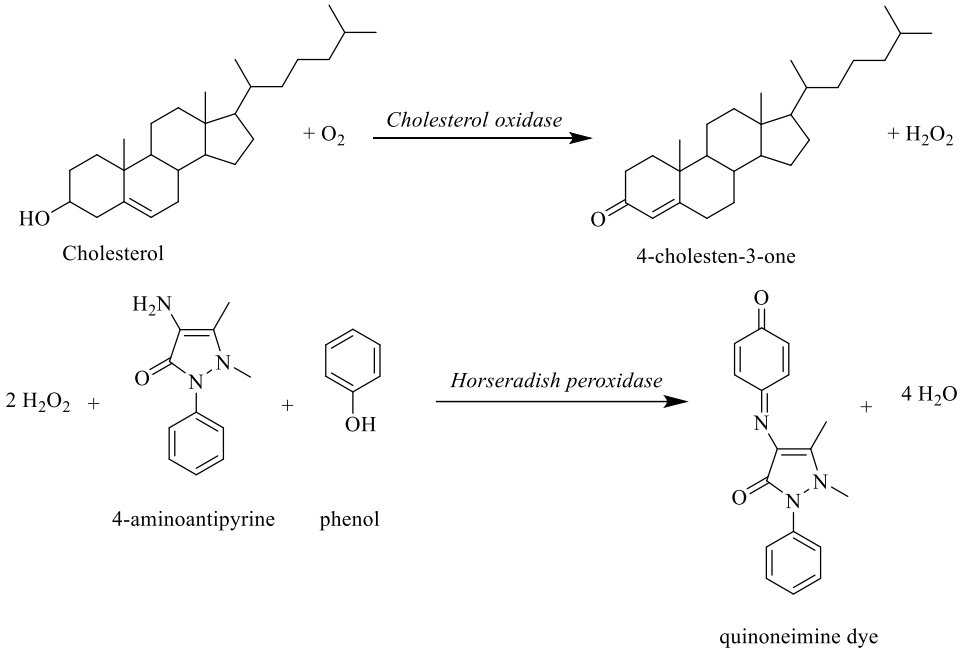


Figure 5-1. Reaction of cholesterol oxidase enzyme activity (modified from Allain et al., 1974)

To discuss with other publications, *Lactobacillus helveticus* CD6 (Ahire et al. 2012) also found cholesterol oxidase enzyme with enzyme activity at 68 U/mg from intracellular extract. This strain was one of the LAB that was isolated from local fermented milk product (Ahire et al. 2013) that utilised cholesterol molecule to be empty within 42 h of fermentation in MRS broth supplemented with 1 mg/ml cholesterol. Furthermore, the recombinant LAB from *L. plantarum* NCL21 (Kiatpapan et al. 2001) as 4.6 mU/mg of protein, while this recombinant strain contained with unsuitable promotor to drive cholesterol oxidase gene. However, this recombinant strain was performed enzyme activity with a lower activity than ENM104 strain in this present study around two folds. While *Enterococcus hirae* Mil-31 (Yehia et al. 2015) isolated from local dairy products in Egypt exposed cholesterol oxidase enzyme activity from culture supernatant at 53.30 ± 0.02 U/mg of protein. This publication detected 4-cholesten-3-one using method that slightly differed from this present study. Another possibility about cholesterol degradation is cholesterol conversion to coprostanol by cholesterol reductase and $NADH+H^+$ that was reported in some LAB as well (Lye et al. 2010). However, we did not study this enzyme in ENM104 strain, so it should be further investigated.

By the way, bile salt molecules are derivatised from cholesterol as the substrate. Then some functional groups on steroid backbone are still located in the same position as the prototype backbone. With the activity to convert cholesterol molecule to 4-cholesten-3-one by cholesterol oxidase enzyme and some activity to deconjugate bile salt to cholic acid and deoxycholic acid backbone in *P. pentosaceus* ENM104, it is possible that bile salts might be also converted with this enzyme in the same position to change to other metabolites and inserted between the somewhere of cholesterol-degrading pathway. Thus, bile salts are repressed to enter to enterocyte cell if pili surface of intestinal tract is colonised with this LAB strain. However, this conversion should be proven in advance.

Regarding the results of this research, it was proved that *P. pentosaceus* ENM104 helped to improve cholesterol-lowering situation using various mechanisms. This strain was able to accumulate cholesterol to inside the cell before degradation. Then cholesterol was degraded by cholesterol oxidase by conversion to intermediate molecule of cholesterol-degrading pathway. Moreover, this strain produced bile salt hydrolase enzymes to deconjugate bile salts which related to the main structure of cholesterol molecule to be broken and unabsorbable for blood circulation. It is possible that this strain might have other mechanisms such as production of short-chain fatty acids and outer hydrophobic compartment of cell absorption that should be further investigated.

8. Tolerance to hydrogen peroxide

Both tested LAB strains (Figure 4-14) did not have catalase enzyme for change hydrogen peroxide to oxygen and water. Oxygen was one of the radical forms to damage bacterial cells. Possibly, this experiment was related to the test for some antioxidant compound production from the bacterial cells to cope with oxygen radical form that could be considered altogether with antioxidant activity experiment. Then the different genus of LAB might differ in the activity to against some radical forms. To compare this work (Gregory and Fridovich 1974), SPS109 strain tolerated hydrogen peroxide longer than the subscribed work that previewed only 90 min toleration. However, concentration of hydrogen peroxide might give the result in the different way, representing 1 mM in our work and 2 mM in subscribed work.

Some specific LAB were found some extraordinary catalase to deal with oxygen radical scavengers (Gregory and Fridovich 1974). Cyanide-sensitive hemoenzyme was established when hematin was contained in the medium that azide and cyanide-insensitive catalase was generated condition of no exogenous hematin. Another one, *L. plantarum* T-1403-5 was found the combination type of azide-sensitive and insensitive catalase in hematin containing medium and *L. plantarum* NZ-48 was found only azide-sensitive catalase in hematin supplementation. However, from this publication, it was hardly related with some extraordinary catalases that were just found only rarely strain of *L. plantarum* (Gregory and Fridovich 1974).

9. Determination of antioxidant activity and total phenolic content

9.1. Comparison of authentic compounds to determine antioxidant activity

Trolox is used as the main authentic compounds to compare amongst the methods used for determination of antioxidant activity in this study (Figure 4-15). This is because the popularity to use as the standard authentic sample in various methods. Besides, trolox shows the consistent reaction with both dissolving in hydrophobic and hydrophilic solvent (Schaich et al. 2015). Antioxidant activity of gallic acid and L-ascorbic acid showed a similar trend with DPPH and ABTS; however, the ORAC result was vice versa. Feasibly, the antioxidant activity depends on the authentic molecule structure like gallic acid which composes of less photon donator than L-ascorbic acid. While DPPH and ABTS reactions showed the results in different levels. Nevertheless, ORAC result displayed less L-ascorbic acid antioxidant activity than gallic acid owning to peroxyl radical production that reacted for some suitable antioxidant molecules (Schaich et al. 2015). Trolox was used as an authentic candidate due to its analogue of vitamin E or α-tocopherol. Thus, it was easy to compare in food application. Furthermore, trolox as an authentic compound was used for antioxidant activity of different methods; however, many publications were used other authentic compounds to discourse. For example, L-ascorbic acid was normally used as authentic compound for DPPH method. To be compared between the detection methods (Figure 4-16), one authentic compound was used as main substance for comparison to be easy to obviously understand. In this experiment, other authentic compounds were

additionally studied to compare with other publications that were included with L-ascorbic acid and gallic acid.

9.2. Antioxidant activity and TPC of selected LAB in MRS medium

Antioxidant activity using ORAC method was greater than the others because of sensitivity of the detection method. As an obvious example, ORAC method detected the activity assessed with the smaller number of free-radical functional group than the larger one which was DPPH and ABTS methods. Thus, the antioxidant activity from the same sample resulted in different ways amongst ORAC and the others. Possibly, bacterial fermentation in ORAC generated better result after 48 h incubation that might be related with metabolites produced after cell growth (Figure 4-17). Moving to ABTS and DPPH methods, the results between two detection methods were not significantly differed. Feasibly, some metabolites that had the effect for an increase of antioxidant activity were established after during the growth phase.

The decision in which methods is better depending the extracts being detection. For instance, DPPH is suitable only polar extracts and not supporting for carotenoids, but ABTS can react with all lipophilic and hydrophilic compounds in both lipophilic and hydrophilic solvents; moreover, ABTS is fast and higher sensitivity than DPPH method (Martysiak-Zurowska and Wenta 2012). Thus, there are the answer why various detectable methods are allowed. To discuss between two selected LAB strains including ENM104 and SPS109, ENM104 strain contributed to generate antioxidant activity better than SPS109 strain; however, there was no significant difference.

From TPC result, the content of phenolic compounds between two selected LAB strains was not significantly differed; however, the fermentation at 24 h provided better result than the other incubation times. An increase of TPC result might be the production of some phenolic acid compounds, especially 3-phenyllactic acid and 4-hydroxyphenyllactic acid that have roles for antioxidant activity and also antimicrobial agents (Mu et al. 2012; Yu et al. 2015). Hence, at least two phenolic acid compounds should be further investigated.

10. Application of potent LAB strains as starter(s) for fermented dairy products

Pasteurised milk purchased in convenient stores was selected to perform this experiment due to economical, convenient, and easy to functionalise for food industry. Then this milk was fermented by selected LAB strains, cholesterol-lowering activity ENM104 and GABA-producing SPS109, either as single culture or co-culture fermentation by compared with control set as no inoculation. After finishing of milk fermentation, curd was generated from acid production by both selected LAB strains. Various parameters were used to detect for milk fermentation. During fermentation, pH was detected in term of growth of both contestant strains because LAB can produce lactic acid as found that SPS109 was better producer than ENM104 in both fermented products (milk and whey). This reflects to acid content in SPS109 set was greater than ENM104 set. However, co-culture set showed the greatest data for both pH and acid content indicating their synergistic in acid environment. For pH in fermented whey, the initial pH was displayed lower than the initial pH of milk as citric acid was used for casein agglutination in making whey. However, to improve the condition of whey fermentation, initial pH of whey should be adjusted to be 6.70-6.90 that is suitable pH for growth of both LAB selected strains.

Whey was selected for this research in order to solve the overwhelming by-product from dairy industry, especially cheese factory and also to add value of this by-product. This by-product can cause for some environmental problems as biological oxygen demand (BOD) in a range of 32,000 - 60,000 mg/L and 60,000 - 80,000 mg/L for chemical oxygen demand (COD) in cause of no treatment before discharging (Cheryan 2000). To find attractive alternative way of use, functional food production is of great interest; therefore, in this study, whey was selected to explore as a new functional beverage. Moreover, whey was compared with fermented milk because both are dairy products.

To count the number of bacterial populations in a co-culture set, new medium was developed based on sugar fermentation and colour to indicate the differences between *Pediococcus* and *Lactobacillus* (Figure 5-2) (Topic 17, Appendix A). To explain, MRS (De Man et al. 1960) was modified by using sucrose instead of glucose as *Pediococcus* sp. like ENM104 cannot utilise sucrose, but *L. plantarum* i.e. SPS109 can consume this sugar (Table A-1). Colour indicator was performed by

bromocresol green due to obvious identification amongst colour tone from bacterial generation. To enlighten, protein in the medium ingredients was the first-choice selection in *Pediococcus* sp. because sucrose cannot be fermented by this strain; meantime, sucrose can be provoked in the counterpart strain. Thus, colour was informed to be light blue with pin-point colony from slow growth colonies in *Pediococcus* sp. Conversely, the indicator was turned from bluish to yellowish tone in *Lactobacillus* sp. from sucrose fermentation.

Consideration on the viable cells of LAB in both fermented milk and fermented whey, SPS109 strain was better than ENM104 in both single and co-culture in milk and whey fermentations. *P. pentosaceus* ENM104 gave the negative result for lactose fermentation (Table 4-8), while this sugar is the major sugar in milk at 4,810 mg in 100 grams (USDA 2019a). However, *L. plantarum* SPS109 can ferment. Hence, SPS109 consumed lactose and casein, while ENM104 consumed only casein or soluble amino acids. This made a tiny growth of ENM104 strain in a co-culture set compared with SPS109 strain. This indicates a competition for nutrients, particularly energy source between both starter cultures and SPS109 was more effective strain. This is confirmed as no significant difference was observed for population of SPS109 strain as single culture or in co-culture. In addition to lactose fermentation, SPS109 strain was more acid tolerance than ENM104 strain (Figure 4-7). Therefore, ENM104 growth did not increase after 24 h of fermentation.

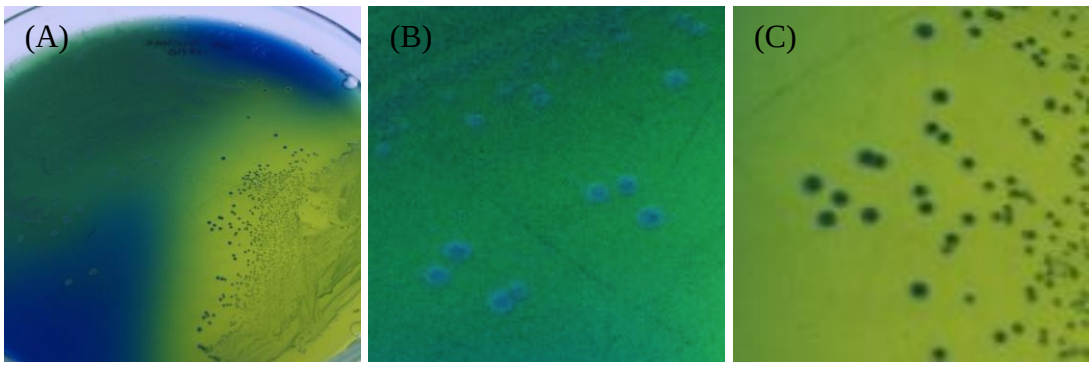


Figure 5-2. (A) *Pediococcus* and *Lactobacillus* in modified MRS agar to distinguish between (B) bluish colonies of *Pediococcus* sp. and (C) yellow-greenish colonies with yellow zone of *Lactobacillus* sp.

As cholesterol oxidase enzyme started to increase from 48 to 72 h from the substantiated results in MRS medium (Table 4-15). This led to the detection of cholesterol in fermented milk and whey using HPLC-DAD. To describe, HPLC with the regression curve concentration range at 0.2-200 µg/ml provided a lower detection limit than spectrophotometric method that the detection limit is in a range of 10 – 200 µg/ml of cholesterol standard in the preliminary estimation (data not shown). Another reason, the unstable reaction to construct the colour with o-phthaldialdehyde (OPA) reagent was observed. Absorbance of reaction fast increased during the measurement time, so it was hard to confirm how cholesterol reduced for cholesterol-lowering result in milk. Cholesterol content reduction in fermented milk was also found in corresponding fermentation time with cholesterol oxidase production at 15.71 ± 0.64 and 10.98 ± 3.80 percent of ENM104 and co-culture sets compared with control set. Plausibly, this enzyme might have a role for this cholesterol content reduction. For fermented whey, the cholesterol content in the normal whey was in a range of 4-11 µg/ml; however, with a low content of cholesterol, the detection might have some problems on amount accuracy of detection as the result was fluctuated. Moreover, after whey was autoclaved, the heat destructed the structure of β-lactoglobulin to be more agglutination (Kilara and Vaghela 2018). The last factor might be possible to perform the fluctuated result. For cholesterol reduction in whey, cholesterol oxidase declined the content down, especially in ENM104 and co-culture sets with corresponding time of enzyme production.

The difference of cholesterol content between fermented milk and fermented whey, it was clear that cholesterol dissolved rather in lipid compartment of milk than casein with the substantiated information from nutrition facts showing the cholesterol contents around 100 µg/kg in milk whole fat (USDA 2019a), 20 µg/kg in skim milk (USDA 2019b) and 10 µg/kg in acid-precipitating whey (USDA 2019c). From the results, ENM104 strain degraded cholesterol by cholesterol oxidase enzyme in milk and whey to decrease around 15 and 55 percent, respectively. The decrease of cholesterol in milk was similar to cholesterol decrease in MRS due to the nutrient abundant to result for cell proliferation. This nutrient abundant contained in whey with the composition of low cholesterol content in whey reflected for higher cholesterol

degradation in ENM104 set around 55 percent that decreased by 2.92 ± 0.70 µg/ml which was lower than in milk by 22.13 ± 4.29 µg/ml or around 15 percent.

There are several reasons that described for the fluctuated results (Figure 4-20 and Figure 4-25). Firstly, bacterial cells could not separate out in milk fermentation because milk is the colloid particle composing from casein protein. When fermented milk was centrifuged, casein was dropped down to the bottom altogether with cell pellets. This case referred to cholesterol accumulation in their bacterial cells. Secondly, non-homogeneous sample as fermented milk was difficultly gathered. Acid production from LAB fermentation is one of the interfering factors. Casein is aggregated altogether in low pH condition at isoelectric point of casein protein around 4.60 (Kilara and Vaghela 2018). When the sample had aggregated altogether, sampling by pipette was difficult to estimate for accuracy although sample was vigorously mixed before sampling. However, it can be concluded that cholesterol oxidase enzyme was produced to reduce cholesterol with the strong support from the enzyme production in MRS medium containing cholesterol.

Moving to topic of glutamic acid and GABA contents in fermented milk and fermented whey, it should be noted that ENM104 could not produce GABA, while SPS109 is a GABA-producing strain (Figure 4-3). For fermented milk, glutamic acid content in SPS109 and co-culture sets reduced by 13.13 ± 3.86 and 16.02 ± 2.93 percent from the start to the end of the fermentation. However, in the fermented whey, glutamic acid content with similar treatments as above decreased by 51.62 ± 0.69 and 52.56 ± 0.44 percent, respectively (Table 4-16 and Table 4-17). These results were supported by SPS109 strain that also reduced glutamic acid at 38.41 ± 0.04 percent (Table 4-6). The reasons to support the different conversion efficiencies are provided in the Table 4-16 and Table 4-17. Firstly, it could be possible that the number of cell growth related with a lower efficiency. Bacterial growth in MRS medium of SPS109 strain at roughly 8.93 log CFU/ml (Table 4-12) was higher than in milk and whey fermentation at 8.29 ± 0.01 and 8.37 ± 0.05 log CFU/ml, respectively. Secondly, glutamic acid contents in MRS, milk and whey was different levels. Thirdly, the most conceivable reason related to casein interference factor in milk overlaid the bacterial growth to be greater because viable cells was not much different between milk and whey fermentation, but the reduction of glutamic acid content was huge in sets of incubated SPS109. Lastly, the

feasible factor related with the releasing of glutamic acid molecule from casein peptide. Free glutamic acid in whey part might contain in a tiny level that relied on proteolysis activity of bacteria to break down casein molecule to release glutamic acid during the fermentation. Comparing between fermented milk and fermented whey, the initial glutamic acid contents was not huge difference as whey was made from casein precipitation in milk. Then glutamic acid was found in whey partition, and this result supported about casein interference in milk and whey fermentation.

For GABA production, the reaction was proceeded as shown in the Figure 2-10. For SPS109 and co-culture sets in fermented milk and fermented whey, free glutamic acid in whey part was used as a substrate to produce GABA because some GABA content was found by bacterial fermentation, and GABA content in fermented whey reached the peak at 24 h of fermentation. One possible reason to support was nutrient limitation in whey as cells used the nutrients for providing energy source. Then in the first 24 h fermentation, lactose was used for bacterial growth to cause high acidity. Then free glutamic acid was converted to GABA by glutamic decarboxylase (GAD) for cell survival under acidic stress (Barla et al. 2016). To compare between milk and whey fermentations, no difference was shown in the initial GABA concentration because whey portion from fermented milk was received from acid precipitation of casein that was similar with fermented whey. Besides, the loss of glutamic acid content with the comparison altogether with MRS fermentation as above, the maximum mole loss in co-culture set as an example was belonged to fermented whey around 52 percent (Table 4-17), fermented milk around 16 percent (Table 4-16) and MRS medium for less than 1 percent (Table 4-6). This suggests that almost all glutamic acids in fermented whey might be used for growing cells that was not for GABA production.

To compare the GABA content both fermented milk and whey by % conversion efficiency (Table 4-16 and Table 4-17) and GABA concentration (Figure 4-21 and Figure 4-26), fermented milk displayed lower content of GABA production than the counterpart with significant GABA increase from the initial content. This information is supported by casein interference because the differences between milk and whey in both experiments as casein micelles might interfere the efficiency of bacterial enzyme. Moreover, bacterial cells might be attached by casein micelles that impulse to hard for free glutamic acid accession.

To compare with other publication, GABA-producing strain named *L. plantarum* NDC75017 was used in sterilised milk fermentation supplemented with 80 mM of MSG and 18 µM of pyridoxal-5-phosphate as coenzyme of glutamic decarboxylase enzyme at 36 °C for incubating temperature to establish yoghurt (Shan et al. 2015). In this yoghurt, GABA was produced from the fermentation at 2,892.30 ± 14.70 µg/g of yoghurt. From this evidence, it is supported that LAB can produce GABA in the dairy fermentation. Another publication, fermented milk using pasteurised milk without MSG addition with the fermentation of four LAB including *Lactobacillus delbrueckii* subsp. *bulgaricus* DPPMALDb5, *Streptococcus thermophilus* DPPMAST1, *L. paracasei* 15N and *L. plantarum* C48 was altogether generated GABA from 43.8 ± 1.8 µg/ml of the initial incubation to 65.1 ± 0.6 µg/ml of 30 days of fermentation (Servili et al. 2011). This meant that GABA-producing LAB can convert free glutamic acid in milk to GABA. GABA concentration in our research was lower roughly 10 folds because milk was prepared by heating before inoculation to have more viscosity. Another support is milk collection which depends on collecting area and cow's strains. However, both fermentation increased GABA content and decreased glutamic acid.

Inhibition of ACE activity with bioactive peptides from which proteolysis activity of bacteria had a role, proteolysis is related with casein digestion to various types of peptides. For the fermented milk, SPS109 strain might be better than ENM104 strain for proteolysis activity (Figure 4-20 and Figure 4-25) that related with ACE inhibition because bioactive peptides from those digestions interrupted ACE enzyme (Hagi et al. 2016). However, only specific peptides can struggle with ACE activity. For instance, the following dipeptides including Leu-Pro, Val-Pro, Ala-Pro, Pro-Pro, Val-Leu and Tyr-Pro and tripeptides such as Val-Pro-Pro. For the collaboration of both strains achieved the greatest result at 72 h with dominant activity by SPS109 (Figure 4-20 and Figure 4-25). Surprisingly, the activity was not raised in exponential phase of bacterial growth. Probably, it is relevant with the use of sugar fermentation for cell growth. For fermented whey, the result was familiar with the counterpart that co-culture set was the most responsive for ACE inhibition; however, the percentage range of inhibitory was much lower. These depended on casein precipitation as casein in whey was eliminated with greater amount than milk in the sample preparation step. These reflect to the percentage of ACE inhibition. Some

evidences supported that GABA-containing diets from GABA-producing bacteria directly had a relationship with ACE inhibition activity owning to hypertension improvement in human body of lacto-tripeptide including Val-Pro-Pro from specific proteolysis of casein molecules (Hagi et al. 2016).

Antioxidant activity results in fermented milk and whey were compared (Figure 4-22 and Figure 4-27) and resulted that the activity was higher in fermented whey for DPPH than fermented milk, but not quite difference in ABTS method. However, the result of ORAC and TPC in fermented milk was higher than fermented whey. Possibly, casein molecules and some amino acids from bacterial proteolysis were impulse these activities to be higher. Some tiny power of hydroxyl group in antioxidative compounds were produced that can be detected by ORAC method. However, the activity in fermented milk was differed from MRS medium, especially at 48 and 72 h. Probably, in MRS medium, it was relevant with secondary metabolite production after sugar consumption, but in fermented milk, proteolysis was relevant. Then antioxidants might be peptide molecules in fermented milk. This encouraged that the role of bacterial fermentation might increase some phenolic and flavonoid compounds, particularly a better result in *P. pentosaceus* ENM104 for antioxidative compounds production than *L. plantarum* SPS109, but for total phenolic content, the results in both fermented milk and fermented whey were exploited in SPS109 and co-culture sets showing the better results; however, these increases should be proved to substantiate by compound identification to detect that these potent compounds were generated from bacterial fermentation or not. Various methods were used to compare the antioxidant activity because some extracts are suitable for some detection methods (Martysiak-Zurowska and Wenta 2012). This suggests that our results in dairy products by ABTS method differed from other extracts detected by DPPH. It could be explained that various detection methods reflect for hydrophobic and hydrophilic antioxidant molecules in fermented milk and whey.

For the increase of total phenolic content in both fermented milk and fermented whey, it was possible that both strains might produce phenolic acid compounds during fermentation. This is in agreement with Vázquez et al. (2015) who reported that cattle feeding especially grasses and some herbs impact for phenolic compounds found in cattle milk. Additionally, 3-phenyllactic acid and 4-

hydroxyphenyllactic acid are the compounds produced in various types of LAB strains, especially *P. pentosaceus* and *L. plantarum* resulting for antimicrobial compounds and also increasing total phenolic content (Mu et al. 2012; Yu et al. 2015). For instance, 3-phenyllactic acid was found in *P. pentosaceus* SK25 by increasing from 17.0 ± 3.2 to 47.2 ± 5.3 mg/L in fermented milk (Yu et al. 2015). Moreover, *L. plantarum* 1081 produced 3-phenyllactic acid up to 5.2 ± 0.5 mM in MRS broth (Gerez et al. 2010). Hence, those phenolic compounds in the present study should originate from grass and increased in both fermented milk and fermented whey by LAB starters. However, the insight study of antioxidant compounds should be performed to answer which compounds were generated in milk/whey fermentation.

Overall results, the role of both starter in co-culture set of both fermented milk and fermented whey demonstrated that *P. pentosaceus* ENM104 was the main organism to reduce cholesterol by combination of cholesterol bioaccumulation, followed by degradation using intracellular cholesterol oxidase and deconjugation of bile salts. Another strain, *L. plantarum* SPS109, helped to convert glutamic acid to GABA as neurotransmitter molecule. With both combination strains in fermented milk and whey, proteolytic activity was synergistically fermented to increase ACE inhibitor as well as antioxidant activity and total phenolic content. However, the evaluation of the product's satisfaction should be manipulated in further experiment.

Interestingly, some strains of *Pediococcus* spp. can produce pediocin as self-bacteriocin production (O'Bryan et al. 2015), so it should be worth to investigate the bacteriocins from *P. pentosaceus* ENM104 for providing this benefit to improve dairy product preparation in which this research used stream-sterilisation. Besides, phenolic compound production from *Pediococcus* spp., especially 3-phenyllactic acid acting like antimicrobial agent to destroy pathogenic bacteria (Mu et al. 2012; Yu et al. 2015). Thus, pediocin and phenolic acid usage might be used in form of direct bacterial culture to avoid pasteurisation or sterilisation for protection of essential nutrients in milk/whey. With expectation that the use of both starters to improve raw materials and fermentation might be applied with other industrial starters especially in fermented dairy products such as yoghurt and acid milk to improve taste, aroma, texture, colour, and consistency to be more satisfied amongst consumers.

CHAPTER 6

CONCLUSIONS

It can be concluded that both selected LAB strains are potential probiotic bacteria to use in food industry for functional food production within various aspects of their properties, especially cholesterol-lowering activity of *P. pentosaceus* ENM104 and GABA-producing activity of *L. plantarum* SPS109 with the supporting evidence as follows. Firstly, both selected LAB strains were isolated from fermented foods, so they are 'generally recognised as safe (GRAS)' to be approved to use as the starter cultures owning to promoting of consumer's health. Secondly, both LAB particularly ENM104 strain produced cholesterol oxidase enzyme to degrade cholesterol to 4-cholesten-3-one, while SPS109 strain generated GABA from glutamic acid. Thirdly, they were proved *in vitro* for their biological barrier in GI tract with the high survivors for acting as probiotics. Lastly, they generated specific peptide chain to inhibit ACE, while they produced antioxidative compounds to reduce free radicals. For application, both LAB strains were used as starters either as single or co-culture for milk and whey fermentation to achieve functional milk and whey products. It was found that they improved milk and whey in fermented milk and fermented whey with lower cholesterol but high in GABA and ACE inhibitor including antioxidants. However, sensory evaluation should also realise amongst the people satisfaction for merchandised product, and this should be conducted in future research.

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APPENDIX

APPENDIX A

Preparation of culture media and reagents

1. de Man Rogosa and Sharpe (MRS) medium broth

MRS medium (Merck, Germany) based on (De Man et al. 1960) with the following formula below.

Peptone from casein	10.00 g
Meat extract	8.00 g
Yeast extract	4.00 g
D(+)-Glucose	20.00 g
Di-potassium hydrogen phosphate (K_2HPO_4)	2.00 g
Polysorbate 80	1.00 g
Di-ammonium hydrogen citrate	2.00 g
Sodium acetate (CH_3COONa)	5.00 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.20 g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.04 g

pH 5.7 ± 0.2 in 1 L of distilled water

This medium is weighed 52.20 g to dissolve in 1 L of distilled water before stream-sterilisation at 121 °C for 15 min.

2. de Man Rogosa and Sharpe (MRS) medium agar

MRS medium broth	52.20 g
Agar	15.00 g

pH 5.7 ± 0.2 in 1 L of distilled water

This medium is weighed 52.20 g to dissolve in 1 L of distilled water and add 15 g of agar before stream-sterilisation at 121 °C for 15 min.

3. MRS agar supplemented with 0.4 mg/ml bromocresol purple for LAB isolation

MRS medium broth	52.20 g
Agar	15.00 g
Bromocresol purple	0.40 g

pH 5.7 ± 0.2 in 1 L of distilled water

This medium is weighed 52.20 g to dissolve in 1 L of distilled water and add 15 g of agar before stream-sterilisation at 121 ℃ for 15 min.

4. LAB basal medium

This medium is modified (De Man et al. 1960; Yehia et al. 2015) by the following formula.

Yeast extract	1.00 g
di-Potassium hydrogen phosphate (K_2HPO_4)	2.00 g
Ammonium sulphate ($(NH_4)_2SO_4$)	2.00 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.20 g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.04 g

pH 7.0 ± 0.2 in 1 L of distilled water

Another solution is dissolved in distilled water before mixing with the above part. Subsequently, pH adjustment and stream-sterilisation at 121 ℃ for 15 min are authorised in the following time.

5. LAB basal medium supplemented with 100 µg/ml cholesterol

This medium is provided for primary screening and tertiary screening as treatment set for cholesterol-lowering LAB selection.

Solution A: LAB basal medium (Topic 4, Appendix A)

Solution B: Cholesterol reagent

Polysorbate 80	1.00 g
Cholesterol	100 mg

To prepare, solution B is weighed and dissolved in a pint of distilled water before ultra-sonication at 50% amplitude with 15 s turning-on and 5 s turning-off in 10 min. Solution A is dissolved in distilled water before mixing with the above part. Subsequently, pH adjustment and stream-sterilisation at 121 °C for 15 min are authorised in the following time.

6. LAB basal medium without yeast extract supplemented with 100 µg/ml cholesterol

This medium is modified (De Man et al. 1960; Yehia et al. 2015) by the following composition.

Solution A: LAB Basal medium without yeast extract

di-Potassium hydrogen phosphate (K_2HPO_4)	2.00 g
Ammonium sulphate ($(NH_4)_2SO_4$)	2.00 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.20 g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.04 g

pH 7.0 ± 0.2 in 1 L of distilled water

Solution B: Cholesterol reagent

Polysorbate 80	1.00 g
Cholesterol	100 mg

To prepare, solution B is weighed and dissolved in a pint of distilled water before ultra-sonication at 50% amplitude with 15 s turning-on and 5 sec turning-off in 10 min. Another solution is dissolved in distilled water before mixing with the above part. Subsequently, pH adjustment and stream-sterilisation at 121 °C for 15 min are authorised in the following time.

7. LAB basal medium supplemented with 100 µg/ml cholesterol-PEG600

This medium is followed from the above LAB basal medium supplemented with cholesterol, but the cholesterol source is replaced to cholesterol-PEG600 as water-soluble cholesterol to be easy for analytical process. The composition is provided as given below.

Solution A: LAB basal medium (Topic 4, Appendix A)

Solution B: 10 mg/ml cholesterol-PEG600 solution

Cholesterol-PEG600	100 mg
Deionised water	10 ml

For the preparation of this medium, solution A, all chemicals are dissolved in distilled water followed by pH adjustment and stream-sterilisation is applied. Moving to the solution B, the chemical is dissolved in deionised water and sterilised with 0.20-µm-sterilised filter-membrane (Sartorius, Germany). To produce this medium, solution B was aseptically added to solution A to make final cholesterol concentration as 100 µg/ml.

8. Medium for γ-aminobutyric acid determination

8.1. MRS medium supplemented with 20 mg/ml MSG

This medium is modified from (Ratanaburee et al. 2013b) with the following composition.

MRS broth medium	52.20 g
Monosodium glutamate (MSG)	20.00 g

pH 5.7 ± 0.2 in 1 L of distilled water

The preparation of this medium is dissolved the above chemical in 1 L of distilled water. Stream-sterilisation at 121 °C for 15 min was allowed.

8.2. GYP medium supplemented with 20 mg/ml MSG

This medium is followed from (DSMZ 2007) as below.

Glucose	20.00 g
Yeast extract	10.00 g
Peptone	10.00 g
Sodium acetate (CH_3COONa)	10.00 g
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.20 g
Manganese sulphate monohydrate ($MnSO_4 \cdot H_2O$)	0.01 g
Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$)	0.01 g
Sodium chloride (NaCl)	0.01 g
Monosodium glutamate (MSG)	20.00 g

pH 6.8 ± 0.2 in 1 L of distilled water

Stream-sterilisation at 121 °C for 15 min is allowed of this medium.

9. Macromolecule hydrolysis medium

9.1. 2 g/L starch agar medium

Meat extract	3.00 g
Peptic digest of animal tissue	5.00 g
Soluble starch	2.00 g
Agar	15.00 g

pH 7.2 ± 0.1 in 1 L of distilled water

All ingredients are mixed and melted with microwave. 25 ml of melted agar is placed each screw cap tube to control the assayed volume before stream-sterilisation at 121 °C for 15 min.

9.2. 10 ml/L tributyrin agar medium

Peptone	5.00 g
Beef extract	3.00 g
Glyceryl tributyrate	10 ml
Agar	15.00 g

pH 7.5 ± 0.1 in 1 L of distilled water

All ingredients, excepted glyceryl tributyrin are mixed and melted with microwave. Glyceryl tributyrin is added and mixed. 25 ml of melted agar is placed each screw cap tube to control the assayed volume before stream-sterilisation at 121 °C for 15 min.

9.3. 20 ml/L skim milk agar medium

Solution A: Basic medium	
Peptone	5.00 g
Beef extract	3.00 g
Agar	15.00 g
pH 7.0 ± 0.1 in 500 ml of distilled water	
Solution B: Skim milk	
Skim milk	20.00 g
Distilled water	500 ml

Ingredients in solution A is solved as double strength and melted with microwave. 12.5 ml of melted agar is placed each screw cap tube to control the assayed volume before stream-sterilisation at 121 °C for 15 min. Solution B was mixed and sterilised with 121 °C for 5 min autoclaving. Then 12.5 ml of solution B is pipetted to place in solution A. Vagarious mixing needs to make homogeneous solution and pours into Petri dish.

10. Bile salt hydrolase activity medium

10.1. MRS agar medium supplemented with 5 mg/ml GCA

MRS broth	52.20 g
Glycocholic acid (GCA)	5.00 g
Agar	15.00 g

The ingredients are mixed in 1 L of distilled water and sterilised with 121 °C for 15 min autoclave. 25 ml per tube needs for assayed control.

10.2. MRS agar medium supplemented with 5 mg/ml GDCA

MRS broth	52.20 g
Glycodeoxycholic acid (GDCA)	5.00 g
Agar	15.00 g

The ingredients are mixed in 1 L of distilled water and sterilised with 121 °C for 15 min autoclave. 25 ml per tube needs for assayed control.

10.3. MRS agar medium supplemented with 5 mg/ml TCA

MRS broth	52.20 g
Taurocholic acid (TCA)	5.00 g
Agar	15.00 g

The ingredients are mixed in 1 L of distilled water and sterilised with 121 °C for 15 min autoclave. 25 ml per tube needs for assayed control.

10.4. MRS agar medium supplemented with 5 mg/ml TDCA

MRS broth	52.20 g
Taurodeoxycholic acid (TDCA)	5.00 g
Agar	15.00 g

The ingredients are mixed in 1 L of distilled water and sterilised with 121 °C for 15 min autoclave. 25 ml per tube needs for assayed control.

11. Cholesterol-lowering activity testing medium

Medium which is performed to test for cholesterol-lowering activity is MRS broth supplemented with 100 µg/ml cholesterol-PEG600

MRS broth medium	52.20 g
Cholesterol-PEG600	100 mg

Cholesterol-PEG600 is firstly dissolved in deionised water for 10 mg/ml and sterilised by filtration. Finished sterilisation of MRS broth medium is added by cholesterol-PEG600 solution to make the final concentration at 100 µg/ml.

12. Gastrointestinal tract *in vitro* testing medium

Whole media are subscribed (Ratanaburee et al. 2013b) for gastrointestinal tract performing.

12.1. Gastric fluid

MRS broth medium	52.20 g
Pepsin	3.00 g

The ingredients are mixed in 1 L of distilled water before adjusting the pH with HCl to pH 2.0 ± 0.1 and sterilised by autoclave at 121 °C in 15 min.

12.2. Intestinal fluid

MRS broth medium	52.20 g
Pancreatin	1.00 g
Bile salts	3.00 g

The ingredients are mixed in 1 L of distilled water before adjusting the pH with NaOH to pH 8.0 ± 0.1 and sterilised by autoclave at 121 °C in 15 min.

13. 8.5 mg/ml normal saline solution (NSS)

This solution was especially prepared for cell-washing step with the following formula in the below.

Sodium chloride (NaCl)	8.50 g

in 1 L of distilled water

This solution is sterilised by autoclave at 121 °C for 15 min before experimental performing.

14. Reagent for testing of hydrogen peroxide tolerance

14.1. 0.1 M phosphate buffer saline (PBS) pH 7.4

Solution A: 0.1 M K_2HPO_4	
di-Potassium hydrogen phosphate (K_2HPO_4; MW = 174.18 g/mol)	17.40 g
Distilled water	1 L
Solution B: 0.1 M KH_2PO_4	
Potassium di-hydrogen phosphate (KH_2PO_4; MW = 136.08 g/mol)	13.60 g
Distilled water	1 L
Solution C: 0.1 M phosphate buffer saline (pH 7.4)	
0.1 M Phosphate buffer	1 L
Sodium chloride (NaCl)	8.50 g

Solution A and B are mixed under controlling of pH meter to receive the buffer for pH 7.4. Then 1 L of the 0.1 M buffer is applied for saline buffer making in solution C. These ingredients are dissolved altogether before allowing to stream-sterilisation at 121 °C for 15 min.

14.2. PBS supplemented with 1 mM hydrogen peroxide

0.1 M phosphate buffer saline (PBS)	1 L
Hydrogen peroxide (MW = 34.01 g/mol, ρ = 1.11 g/ml)	10.21 ml

PBS is sterilised before adding filtered-sterilised hydrogen peroxide to this reagent with aseptic technique.

15. Sterilised whole milk

Commercial pasteurised milk is purchased from the local convenient shop. 200 ml of milk is measured and contained in large Duran's bottle. Fast exhausting stream sterilisation needs to use for milk sterilisation with 121 °C for 15 min.

16. Sterilised whey

Cottage cheese is selected for whey preparation with by-product from cheese agglutination, and this cheese is easy-to-made household making. The preparation of whey from cottage cheese is followed (Scheme A-1).

Scheme A-1. Whey preparation

Whey preparation

- Measure 1 L of pasteurised milk
- Mix 2.5 g of commercial citric acid with the above milk
- Heat the mixture with 85 °C water bath with no disturbed, placed 15 min
- Filtrate that mixture with gauze and cotton for separation of casein-agglutination and whey compartment
- Use Whatman no.1 paper to filter that whey
- Sterilise that whey with stream-sterilisation at 121 °C for 15 min

17. Modified MRS medium for *Pediococcus* and *Lactobacillus* differentiation

This medium is modified from MRS medium (Merck, Germany), the result of sugar fermentation from API 50 CHL (BioMérieux, France) and heterofermentative-homofermentative differentiated medium (HHD) (McDonald et al. 1987). Based on the theory, *Pediococcus* spp. does not have the capability of some sugar fermentations. Instead of occurring of sucrose fermentation, nitrogen nutrients in the medium composition are used. Then no acid production is generated from nitrogen fermentation, and colouring indicator is not changed from bluish to yellowish colour. On the other hand, *Lactobacillus* spp. can utilise sucrose as carbon source to growth. When sugar fermentation is generated, the colour indicator changes from bluish to yellowish colour from acid production. Table A-1 shows about the difference of sugar fermentation between *Pediococcus* spp. and *Lactobacillus* spp.

Table A-1. Differentiation of sugar fermentation between *Pediococcus pentosaceus* ENM104 and *Lactobacillus plantarum* SPS109 from the result of API 50 CHL (BioMérieux, France)

Sugar	**Amounts (mg/100µl)**	**ENM104**	**SPS109**
D-xylose	1.40	+	-
D-mannitol	1.36	-	+
D-sorbitol	1.36	-	+
Methyl-αD-mannopyranoside	1.28	-	+
D-lactose	1.40	-	+
D-melibiose	1.32	-	+
D-sucrose	1.32	-	+
D-melezitose	1.32	-	+
D-raffinose	1.56	-	+
D-tagatose	1.40	+	-
Potassium gluconate	1.84	-	+

Ingredients of this medium are provided as follows.

Sucrose	10.00 g
Peptone	10.00 g
Yeast extract	5.00 g
Polysorbate 80	1 ml
Di-potassium hydrogen phosphate (K_2HPO_4)	2.00 g
Sodium acetate (CH_3COONa)	5.00 g
Tri-ammonium citrate ($C_6H_{17}N_3O_7$)	2.00 g
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.20 g
Manganese sulphate monohydrate ($MnSO_4 \cdot H_2O$)	0.05 g
Bromocresol green	0.06 g

pH 7.0 ± 0.2 in 1 L of distilled water

All ingredients are mixed and dissolved altogether, excepted polysorbate 80 in 1 L of distilled water. When all ingredients are dissolved, polysorbate 80 is orderly added. Stream-sterilisation needs to sterilise this medium at 121 °C for 15 min. Colour of the medium after finishing is shown in dark-bluish colour.

APPENDIX B

Analytical procedures

1. Gram's staining

1.1. Gram's staining solution

1.1.1. Crystal violet solution

Solution A: Crystal violet	
Crystal violet	2.00 g
Absolute ethanol	20 ml
Solution B: Ammonium oxalate	
Ammonium oxalate	0.80 g
Distilled water	80 ml

Both solutions are mixed and waited for precipitation around 24 h before filtrating with Whatman no.1.

1.1.2. Lugol's iodine solution

Iodine	1.00 g
Potassium iodide (KI)	2.00 g
Distilled water	300 ml

This solution is prepared by dissolving the mass partition in distilled water before storing in brownish glass bottle to protect the light.

1.1.3. Safranin solution

Safranin O	2.50 g
Absolute ethanol	100 ml
Distilled water	900 ml

Safranin O is thoroughly dissolved in absolute ethanol and waited for precipitation for 24 h. Whatman no.1 needs to be used for filtration, and this reagent is more dissolves with distilled water before using.

1.2. Gram's staining procedure

The reagent for Gram's staining can be prepared as the above. For the procedure, it is shown (Scheme B-1).

Scheme B-1. Gram's staining procedure

Isolated colony of bacterial sample

- Smear the isolated colony on glass slide and wait until dry
- Fix the glass slide on the flame in a little portion
- Flood the crystal violet on smearing strain for 1 min
- Discard and wash the dye out
- Flood the Gram's iodine on smearing strain for 1 min
- Discard and wash the dye out
- Decolourise the dye with 95% ethanol for 5-7 s
- Flood the safranin on smearing strain for 30 s
- Discard and wash the dye out
- Wait until the staining to dry
- Observe under microscope (Olympus CX31, Japan)

The result is illustrated for shape of bacteria and colour that classify to Gram's negative in reddish colour and Gram's positive in purplish colour.

2. Catalase test

2.1. Catalase test solution

Hydrogen peroxide (H_2O_2) is concentrated at 30% (w/w). To make the solution at 30 mg/ml hydrogen peroxide solution, procedure is followed in the below.

30% (w/w) hydrogen peroxide (9.79 M; ρ = 1.11 g/ml)	9.01 ml
Distilled water	90.90 ml

The solution after mixing is kept in cold refrigerator to prolong the reagent life with aluminium foil-covered protection.

2.2. Catalase test procedure

The procedure for catalase test is performed (Scheme B-2).

Scheme B-2. Catalase test procedure

Isolated colony on the agar

- Drop 3% (w/v) hydrogen peroxide (H_2O_2) solution on the glass slide
- Pick an investigated colony to mix with H_2O_2 solution
- Observe the bubble production

Catalase-positive result is shown the bubble production, meanwhile, catalase-negative result is not shown any bubble production.

3. Cholesterol determination by spectrophotometer

3.1. Chemical preparation for cholesterol determination

3.1.1. 5.88 M Potassium hydroxide (KOH)

85% Potassium hydroxide (MW = 56.11 g/mol)	38.82 g
Distilled water	100 ml

This chemical is weighed before dissolving in distilled water and adjusting with volumetric flask to be fit as 100 ml.

3.1.2. 500 mg/L o-phthaldialdehyde (OPA) solution

O-phthaldialdehyde (OPA)	50.00 mg
Glacial acetic acid	100 ml

This reagent is freshly prepared before using and should be used for one time per determination.

3.2. Cholesterol-determined preparation

For the cholesterol determined preparation step from the culture, it is followed (Scheme B-3).

Scheme B-3. Culture cholesterol-determined preparation step

5 ml of cultures
- Centrifuge at 5,000 × *g* for 10 min

Cell pellets
- Twice wash with normal saline solution
- Centrifuge at 5,000 × *g* for 10 min
- Discard the liquid part out
- Suspend cell in 1 ml of hexane
- Ultra-sonicate (Sonics, USA) for 60% amplitude with 20 s turning-on and 10 s turning-off cycle with total time 6 min in ice bath
- Centrifuge at 12,000 × *g* for 10 min at 4 °C to discard cell debris
- Apply to cholesterol determination

Culture supernatant
- Apply to cholesterol determination

3.3. Cholesterol determination by spectrophotometry

For the cholesterol determination, this method is referred (Rudel and Morris 1973; Tomaro-Duchesneau et al. 2014) (Scheme B-4).

Scheme B-4. Cholesterol determination by spectrophotometry method

1 ml of sample/standard/blank: 15-ml methanol-washed glass screw cap tube
- Add 1 ml of 5.88 M potassium hydroxide and 2 ml of absolute ethanol
- Close the cap tightly and mix with vortex for 10 s
- Boil at 85 °C for 15 min
- Replace in cold water to reduce temperature immediately
- Add 1 ml of deionised water and 1.5 ml of hexanes
- Vigorous mix with vortex for 1 min and wait for 10 min for clear phase separation

- Pipette 1 ml of hexane layer to 2-ml sizing glass vial
- Evaporate with CentriVap (Labconco, USA) with 40 °C heat-all-time for 30 min or until dry
- Add 1 ml of 500 mg/L OPA solution and 250 µl of concentrated sulphuric acid (H_2SO_4)
- Vigorous mix with vortex for 20 s and wait for 20 min
- Detect the absorbance at 570 nm with spectrophotometer (Thermo Scientific Genesys 10S UV-Vis, the United States)

3.4. Cholesterol standard by spectrophotometer

The standard for cholesterol determination is used cholesterol authentic sample following the concentration (Scheme B-5).

Scheme B-5. Cholesterol-PEG600 standard concentration

Stock solution: 10 mg/ml cholesterol-PEG600

- Weigh 100 mg of cholesterol-PEG600 and dissolve in 10 ml of distilled water
- Dilute 10-fold stock solution from 10 mg/ml to 1,000 µg/ml
- 5 ml of working solution in dilution table shown in the below

Concentration (µg/ml)	**1,000 µg/ml solution (ml)**	**Distilled water (ml)**
200	1,000	4,000
150	750	4,250
100	500	4,500
50	250	4,750
10	50	4,950
5	25	4,975

The regression curve and equation of cholesterol-PEG600 standard curve are displayed in the Figure B-1.

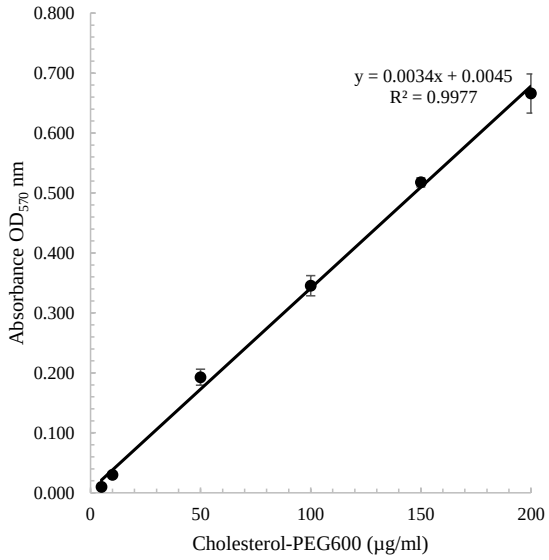


Figure B-1. Regression curve of cholesterol-PEG600 standard with regression equation as y = 0.0034x + 0.0045, R^2 = 0.9977

4. Cholesterol determination by HPLC-DAD

4.1. Chemical preparation for cholesterol determination

4.1.1. Mobile phase

For this determination, mobile phase is used the mixture of three solvents composed of acetonitrile, methanol and 2-propanol following in the below.

Acetonitrile	700 ml
Methanol	300 ml
2-Propanol	100 ml

After mixing the mixture, 0.22 nylon filter is prepared for filtration before using as mobile phase solvent of HPLC.

4.1.2. 50 µg/ml spiked cholesterol in methanol for sample dissolution

Cholesterol (C8667-25G, sigma, USA)	5 mg
Methanol	100 ml

Spiked cholesterol solution in methanol is used for sample dissolution.

4.2. Cholesterol-determined preparation

The determination of the amount of cholesterol is modified the method (Oh et al. 2001) with the procedure (Scheme B-6).

Scheme B-6. Cholesterol determination by HPLC-DAD

Dry matter of cholesterol substance: after evaporation in extraction step of Scheme B-4

- Add 500 µl of methanol spiked with 50 µg/ml cholesterol standard
- Vigorously vortex to dissolve cholesterol dry matter
- Inject to HPLC-DAD (Agilent 1200, USA)

4.3. Cholesterol determination by HPLC-DAD

High-performance liquid chromatography (HPLC; Agilent 1200, USA) with diode array detector (DAD) is performed this determination (Oh et al. 2001). Wavelength is set for sample wavelength at 205 nm with 6 nm of bandwidth and reference wavelength at 500 nm with 6 nm of bandwidth. Sample is injected for 50 µl to the mobile phase composed of the mixture of acetonitrile: methanol: 2-propanol at the ratio 7: 3: 1 in the column of Nucleosil 100-5 C_{18} reversed phase, 720014.40, 250×4.0 mm, 5 µm; Macherey-Nagel, Germany) with thermostat at 30 °C. Cholesterol peak is appeared with the retention time around 9.098 min.

4.4. Cholesterol standard by HPLC-DAD

Authentic sample of cholesterol (C8667-25G, sigma, USA) is performed this standard (Scheme B-7).

Scheme B-7. Cholesterol standard concentration for HPLC determination

Stock solution: 10 mg/ml cholesterol standard

- Weigh 100 mg of cholesterol and dissolve in 10 ml of distilled water
- Dilute 50-fold stock solution from 10 mg/ml to 200 µg/ml
- 1 ml of working solution in dilution table shown in the below

Concentration (µg/ml)	200 µg/ml solution (ml)	Spiked methanol (ml)
200	1000	0
100	500	500
50	250	750
Concentration (µg/ml)	**20 µg/ml solution (ml)**	**Spiked methanol (ml)**
20	1000	0
10	500	500
2	100	900
1	50	950
0.2	10	990

Regression curve is illustrated (Figure B-2).

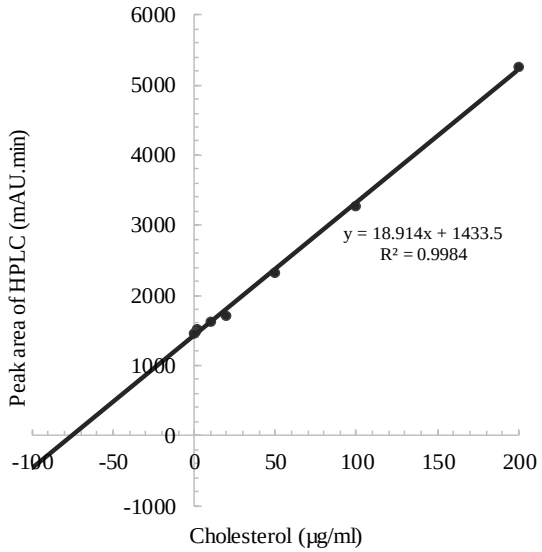


Figure B-2. Cholesterol regression of HPLC determination with regression equation as $y = 18.914x + 1433.5$, $R^2 = 0.9984$

5. Activity determination of cholesterol oxidase-resembled enzyme

5.1. Reagent preparation

5.1.1. Enzyme-reacting mixture

The mixture is subscribed (Ahire et al. 2012; Allain et al. 1974) with the following formula below.

Sodium cholate from bile salt no.3	1.38 g	(64 mM final conc.)
Triton X-100	170 µl	(3.4 ml/L final conc.)
Cholesterol	17.40 mg	(0.9 mM final conc.)
4-aminoantipyrine	14.21 mg	(1.4 mM final conc.)
Phenol	98.82 mg	(21 mM final conc.)
Horseradish peroxidase	75 U	(1.5 U/ml final conc.)

50 ml of deionised water

Sterilised deionised water is placed in 50 ml of sterilised Duran's bottle firstly, followed by Triton X-100 and thoroughly mixed. Sodium cholate is added in the next and well dissolved. The remaining composition is added in the following.

5.1.2. Bradford reagent for determination of protein contents

The reagent is subscribed the formula (Kruger 2003) described below.

Coomassie brilliant blue G250	100.00 mg
95% ethanol	50 ml
85% ortho-phosphoric acid	100 ml

1 L of distilled water

Ingredients are mixed altogether. Finally, reagent colour is previewed as greenish blue tone.

5.2. Procedure of enzyme activity determination

The procedure is modified from the large scale method described (Ahire et al. 2012) with some modification to microtiter plate scale (Scheme B-8).

Scheme B-8. Procedure of enzyme activity determination

Enzyme-reacting mixture

- place 200 µl of enzyme-reacting mixture in a microtiter plate well
- Incubate at 37 °C for 10 min

Sample	**Blank**	**Standard**
20 µl of crude enzyme extract	20 µl of crude enzyme extract solvent	20 µl of each H_2O_2 concentration

- Immediately detect with a microplate reader at 500 nm from 0 to 5 min
- Calculation to find enzyme activity

5.3. Standard solution of H_2O_2

Suitable concentrations of hydrogen peroxide (H_2O_2) from 30% (w/w) or 9.97 M H_2O_2 for enzyme activity standardisation is shown (Scheme B-9).

Scheme B-9. H_2O_2 concentration for enzyme activity standardisation

1 M H_2O_2 solution

- Add 102 µl of 9.79 M H_2O_2 to 898 µl of deionised water
- Dilute 1000 folds of stock solution to 1 mM H_2O_2 solution
- Make the prospective concentration in the following below

H_2O_2		**1 mM H_2O_2 solution (µl)**	**Deionised water (µl)**
Conc. (mM)	**Mass (nmol)**		
0.010	0.2	10	990
0.050	1.0	50	900
0.100	2.0	100	750
0.250	5.0	250	250
0.500	10.0	500	500
1.000	20.0	1,000	0

Regression curve is provided (Figure B-3).

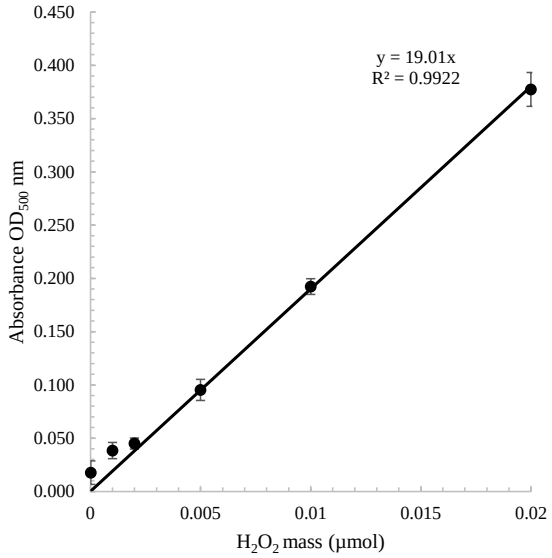


Figure B-3. Regression curve of H_2O_2 (µmol) detected by 500 nm absorbance with regression equation as y = 19.01x, R^2 = 0.9922

5.4. Determination of protein content

For determination of protein content, Bradford method is selected for this determination. (Kruger 2003) with the following (Scheme B-10).

Scheme B-10. Determination of protein content by Bradford method

Bradford solution

- Add 200 µl of Bradford solution
- Place each complement in a microtiter plate

Sample	**Blank**	**Standard**
20 µl of crude enzyme extract	20 µl of crude enzyme extract solvent	20 µl of each BSA concentration

- Detect in 2-60 min after adding the solution with 595 nm of a microplate reader

5.5. Standard solution of protein determination

Suitable concentration range of bovine serum albumin (BSA) for protein determination is shown (Scheme B-11).

Scheme B-11. BSA concentrations for protein determination

5 mg/ml bovine serum albumin (BSA) solution

— Weigh 5 mg of BSA and add 1 ml of deionised water to dissolve

— Make the prospective concentration in the following below

Bovine serum albumin		**100 µg/ml H_2O_2 solution (µl)**	**Deionised water (µl)**
Conc. (µg/ml)	**Mass (µg)**		
20	0.40	200	800
40	0.80	400	600
60	1.20	600	400
80	1.60	800	200
100	2.00	1,000	0

Regression curve of protein determination is provided (Figure B-4).

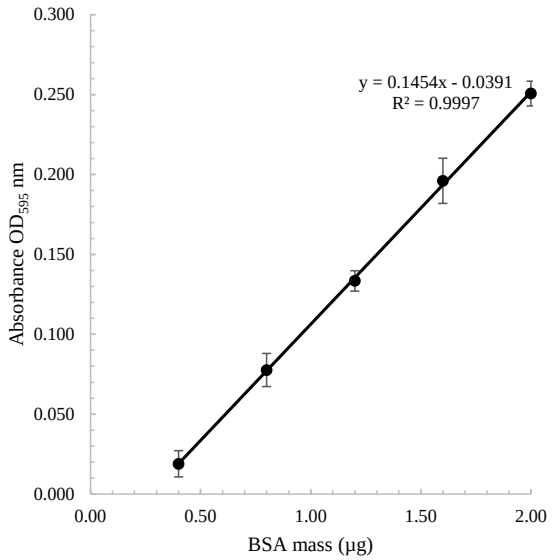


Figure B-4. Regression curve of BSA (µg) detected by 595 nm absorbance with regression equation as $y = 0.1454x – 0.0391$, $R^2 = 0.9997$

5.6. Determination of specific enzyme activity

Specific enzyme activity is defined with the following formula.

$$Specific\ enzyme\ activity\left({}^{U}/_{mg}\right) = \frac{Enzyme\ activity\ (Unit)}{Protein\ content\ (mg)}$$

6. Determination of γ-aminobutyric acid by TLC

6.1. Chemical preparation

6.1.1. 1 mg/ml ninhydrin solution

This solution is used for colour generation of GABA determination from thin layer chromatography (TLC) referred from (Bhushan and Martens 2003).

Ninhydrin	1.00 g
Absolute ethanol	700 ml
Glacial acetic acid	210 ml
2,4,6-trimethylpyridine	29 ml

The chemicals are dissolved altogether and kept in aluminium-covered bottle as light protector. Before using, this reagent is contained in glass spray container and sprayed overall of TLC plate before incubating in 100 °C of hot air oven.

6.1.2. Mobile phase of TLC

The mobile phase to investigate the GABA from the crude sample is used as ratio representing butan-1-ol: glacial acetic acid: deionised water as 4: 1: 1.

Butan-1-ol	200 ml
Glacial acetic acid	50 ml
Deionised water	50 ml

This mixture is provided the total volume as 300 ml. After finishing mixing, this mixture is poured in the glass chamber and closed with glass lid for solvent vaporisation to equilibrium state.

6.2. Procedure of γ-aminobutyric acid determination by TLC

The procedure of thin layer chromatography (TLC) to forecast γ-aminobutyric acid-producing strain is described (Scheme B-12).

Scheme B-12. Procedure of TLC to find γ-aminobutyric acid-producing LAB

3 µl of culture supernatant

- Drop on TLC plate (Silica gel 60 F_{254}, Merck) as Figure B-5
- Dry the spot sample until no liquid containing
- Equally put the TLC plate to mobile phase chamber and close the lid
- Wait until the solvent running to solvent front line around 4:45 h
- Dry the TLC plate until no solvent holding on
- Overwhelmingly spray with 1 mg/ml of ninhydrin solution to the TLC plate
- Incubate the TLC plate in 100 °C for 10 min
- Observe the colour generation on TLC plate and measure retardation factor (R_f) of all standards and samples with the following formula

$$R_f = \frac{distance\ of\ solute\ running}{distance\ of\ solvent\ running}$$

R_f is shown as the unique number of each chemical on chromatography. Then R_f of samples providing the GABA result is displayed the similar number with R_f of GABA standard.

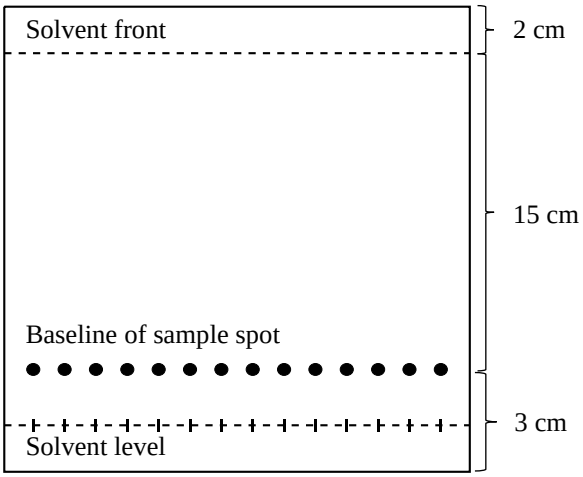


Figure B-5. TLC plate template (Silica gel 60 F_{254}, Merck) for GABA investigation

7. Determination of γ-aminobutyric acid and glutamic acid by HPLC

7.1. Chemical preparation

7.1.1. O-phthaldialdehyde (OPA) reagent for HPLC derivatisation

Solution A: 0.2 M Borate buffer pH 9.9

Solution A-1: 0.2 M Boric acid in 0.2 M KCl

Potassium chloride (KCl; MW = 74.55 g/mol; 0.2 M KCl)	0.37 g
Boric acid (H_3BO_3; MW = 61.83 g/mol)	0.31 g
Deionised water	25 ml

Solution A-2: 0.2 M NaOH

Sodium Hydroxide (NaOH; MW = 40.00 g/mol)	0.20 g
Deionised water	25 ml

Solution B: 5 mg/ml methanolic o-phthaldialdehyde

O-phthaldialdehyde (MW = 134.13 g/mol; OPA)	12.50 mg
Methanol (HPLC grade)	2.5 ml

Solution C: OPA reagent solution

Borate buffer (pH 9.9)	8 ml
Methanolic OPA	2 ml
2-mercaptoethanol	20.00 µl

Solution A-1 and A-2 are mixed altogether with pH adjustment to pH 9.9. Briefly, 25 ml of solution A is appropriately mixed with 20 ml solution B. This buffer is stored in 4 °C for several months. Solution B should be freshly prepared before HPLC derivatisation. Moreover, solution C is combined solution A and B altogether. After mixing the solution, this could be contained in brownish HPLC vial to protect the ambient light and could be mixed 2 h before injection. Life was available for 24 h.

7.1.2. Mobile phase for HPLC

7.1.2.1. 0.2 M Phosphate buffer pH 7.0

Solution A: 1 M K_2HPO_4

Di-potassium hydrogen phosphate (K_2HPO_4; MW = 174.18 g/mol)	43.55 g
Deionised water	250 ml

Solution B: 1 M KH_2PO_4

Potassium di-hydrogen phosphate (KH_2PO_4; MW = 136.09 g/mol)	27.22 g
Deionised water	200 ml

Solution C: 0.2 M phosphate buffer pH 7.0

1 M Phosphate buffer pH 7.0	180 ml
Deionised water	740 ml

Both solutions are thoroughly mixed altogether and adjusted with pH meter to receive pH 7.0 buffer solution. This stock solution is diluted before injection from 1 M buffer to 0.2 M buffer with the procedure as given below. Total volume of solution C is 920 ml buffer and filtrates with 0.22 µm nylon membrane before using for HPLC mobile phase injection.

7.1.2.2. 100% Acetonitrile

This acetonitrile is used as HPLC grade and filtrated with 0.22 µm nylon membrane before HPLC mobile phase injection.

7.2. Procedure of GABA and glutamic acid determination by HPLC

The quantity of glutamic acid and GABA determination is referred from (Populin et al. 2007). HPLC is propelled by Agilent 1200 Series (Agilent, USA) with fluorescence detector (FLD). The detected wavelength is 330 nm as excited wavelength and 440 nm as emitted wavelength. 75 µl of OPA reagent solution is derivatised with 15 µl of sample by in-the-machine combination and 10 times mixing in-the-air condition. The injection must wait for 2 min after mixing for complete reaction. Column is driven by Hypersil ODS C18 sizing as 250 × 4.0 mm; 5 µm particle size with 30 °C controlled temperature (79926OD-584; Agilent, USA) with (A) 0.2 M phosphate buffer pH 7.0 and (B) 100% acetonitrile as mobile phase, and the gradient ratio shows in the Table B-1.

Table B-1. Mobile phase gradient for glutamic acid and GABA quantity determination

Time (min)	**A: 0.2 M phosphate buffer pH 7.0**	**B: 100% acetonitrile**
0	87	13
15	87	13
40	50	50
60	15	85
62	15	85

7.3. Glutamic acid and GABA standard for HPLC quantification

The quantification of glutamic acid and GABA is used glutamic acid (G1251-100G, Sigma-Aldrich, USA) and GABA (A2129-10G, Sigma-Aldrich, USA) as authentic samples. The preparation of combined authentic samples is shown (Scheme B-13).

Scheme B-13. Glutamic and GABA standard concentration

Stock solution: 1 mg/ml glutamic and GABA concentration

- Weigh 100 mg of each glutamic acid and GABA and dissolve altogether in 100 ml of deionised water
- Dilute 100-fold solution to 10 µg/ml combined glutamic acid and GABA
- 1 ml of working solution in dilution table shown in the below

Concentration (µg/ml)	**10 µg/ml solution (ml)**	**Distilled water (ml)**
8	800	200
5	500	500
3	300	700
1	100	900
0.8	80	920
0.5	50	950
0.3	30	970
0.1	10	990

The regression curve and equation of combined glutamic and GABA from HPLC quantified determination are illustrated (Figure B-6).

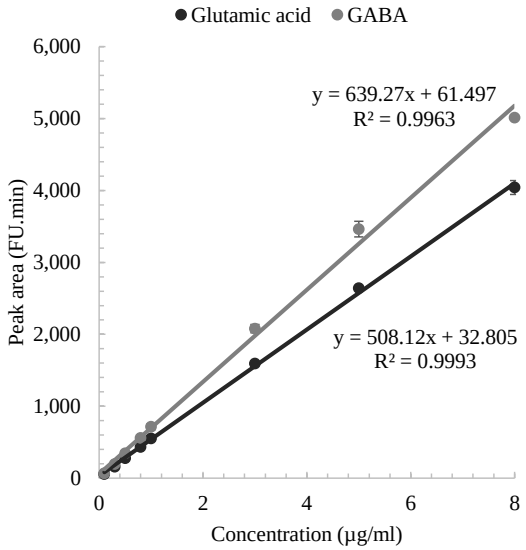


Figure B-6. Glutamic acid regression curve (●) with equation as y = 508.12x + 32.805; R^2 = 0.9993, and GABA regression curve (●) with equation as y = 639.27x + 61.497; R^2 = 0.9963

8. Identification of contestant LAB by biochemical technique

8.1. Reagent for biochemical technique

8.1.1. API 50 CHL medium

This biochemical medium is referred from API 50 CHL (BioMérieux).

Polypeptone (bovine/porcine origin)	10.00 g
Yeast extract	5.00 g
Tween 80	1 ml
di-Potassium phosphate	2.00 g
Sodium acetate	5.00 g
di-Ammonium citrate	2.00 g
Magnesium sulphate	0.20 g
Manganese sulphate	0.05 g
Bromocresol purple	0.17 g

pH 6.9 ± 0.2 in 1 L of deionised water

Stream-sterilisation at 121 °C for 15 min is allowed of this medium.

8.2. Procedure of identification by biochemical technique

This identification is referred the method from API 50 CHL (Ref 50410; BioMérieux, France) with the following procedure (Scheme B-14 and Scheme B-15).

Scheme B-14. Bacterial cell preparation for biochemical test

5 ml of each contestant strains culture in MRS broth medium

- Centrifuge at 5,000 × *g* for 10 min
- Discard supernatant out
- Twice wash with 1 ml of 8.5 g/L normal saline solution
- Suspend those cells in 1 ml of 8.5 g/L normal saline solution
- Adjust those cells to 2 McFarlands or OD_{660} = 0.335 in 10 ml volume
- Remove normal saline solution out by 5,000 × *g* for 10 min centrifuge
- Suspend cells in 10 ml of API 50 CHL medium and thoroughly mix

Scheme B-15. Biochemical test of API 50 CHL (BioMérieux, France)

API 50 CH strip: for carbohydrate testing (Ref 50300; BioMérieux, France)

- Combine the plastic tray by put the distilled water in the water pits and arrange the strip in the tray, respectively
- Add 120 µl of bacterial cell suspension in the medium to each strip well
- Cover the mouth well by 50 µl of paraffin oil
- Incubate the whole tray at 35 °C
- Read the results at 24 h and 48 h incubation time by purplish colour as negative result and yellowish colour as positive result of 0th to 49th well number excepted well number 25th to read blackish colour as positive result
- Transcribe the result by apiweb™ identification software with database (V5.1) programme for bacterial identification

9. Identification of contestant LAB by molecular technique

9.1. Reagent for molecular technique

9.1.1. Gram’s positive difficult-to-lyse reagent enzyme solution

Enzyme solution for Gram’s positive difficult-to-lyse reagent is separately prepared for two parts in the following.

Solution A: Tris-EDTA solution

Tris·HCl (pH 8.0; MW=157.60 g/mol)	0.32 g	(20 mM as final conc.)
EDTA (MW = 372.24 g/mol)	74.40 mg	(2 mM as final conc.)
Triton X-100	1.20 ml	(1.2% as final conc.)

Solution B: Enzyme solution

Solution A	1 ml
Lysozyme	20.00 mg

Solution A is mixed and dissolved in 100 ml of deionised water before stream-sterilisation at 121 °C for 15 min. For solution B, lysozyme is weighed and put in sterilised microtube. 1 ml of solution A is applied to this microtube and thoroughly mixed before already using as molecular solution.

9.1.2. TAE buffer

This buffer is used the commercial product and diluted before using.

Solution A: 50x TAE buffer (Bio-rad, USA)

Tris Base (MW = 121.1 g/L)	121.10 g
Acetic acid	60.50 ml
EDTA sodium salt dihydrate (MW = 372.24 g/L)	372.24 g

Solution B: 1x TAE buffer

50x TAE buffer	20 ml
Distilled water	980 ml

Solution A is purchased as a commercial product. To prepare the working solution, distilled water is sterilised with autoclave at 121 °C for 15 min before adding 50x TAE buffer and thorough mixing.

9.2. Procedure of identification by molecular technique

For molecular identification, stock culture of contestant LAB in -80 °C cryopreservative are activated on MRS agar medium for 24 – 48 h in 35 °C incubation. One colony of each contestant is inoculated in 5 ml of MRS broth medium and incubated for 18 h at 35 °C. The procedure of DNA extraction, amplification and purification is followed (Scheme B-16, Scheme B-17, Scheme B-18, and Scheme B-19). The DNA extraction is followed (Scheme B-16) the procedure from QIAamp® DNA mini kit (Qiagen, Germany).

Scheme B-16. Extraction step of LAB (Qiagen, Germany)

1 ml of contestant culture

- Harvest by centrifuge at 5,000 × *g* for 10 min
- Discard the cell-free supernatant out
- Resuspend the cell pellets in 180 µl of Gram's positive difficult-to-lyse reagent enzyme solution and thoroughly vortex
- Incubated at 37 °C for 24 h in heat box to lyse bacterial cell wall
- Add 20 µl of proteinase K and 200 µl of buffer AL

- Meticulously mix by slowly handled inversion mixing
- Incubate at 56 ℃ for 30 min for undamageable breaking cell membrane
- Add 200 µl of ethanol and slowly handled inversion mixing
- Apply the DNA solution to combined QIAamp Mini spin column and collection tube by slowly pouring
- Centrifuge 6,000 × *g* for 1 min and discard the solution in collecting tube
- Add 500 µl of buffer AW1 to the spin column
- Centrifuged at 6,000 × *g* for 1 min and discard the solution in collecting tube
- Add 500 µl of buffer AW2 to the spin column
- Centrifuged at 17,000 × *g* for 1 min and discard the solution in collecting tube
- Re-centrifuge at 17,000 × *g* for 3 min for eliminate some droplets
- Combine the spin column with 1.5-ml-sizing new microtube
- Add 30 µl of 56 ℃-incubated buffer AE in the spin column
- Incubate for 1 min to release the DNA from the filter membrane out
- Centrifuged at 6,000 × *g* for 1 min
- Store genomic DNA at -20 ℃ for amplification step

Genomic DNA from this extraction should be checked by gel electrophoresis before moving to the next step (Scheme B-17).

Scheme B-17. Gel electrophoresis for DNA checking

Agarose gel preparation

- Add 0.5 g of agarose to 50 ml of TAE buffer for 0.1% (w/v) agarose
- Melt with microwave until gel dissolving around 2 min at 900W
- Release the heat out by cold water until warm at liquid gel state
- Pour in the gel block and wait until gel clotting
- Set gel in TAE buffer of electrophoresis system (Bio-rad, the United States)

DNA loading

- Cut the enough sizing parafilm around 1 × 5 cm and unwrap inside to use
- Drop the reagent as preparing below with MaestroSafe Nucleic Acid loading dye (Maestrogen, Taiwan) as the first and follow by expected DNA

Lane	Expected DNA		loading dye	Total volume
	List	Volume (µl)	(µl)	(µl)
1	1 kb Gene Ruler (Thermo Scientific, 2018)	2	2	4
2	Extracted DNA	5	2	7
⋮	⋮	⋮	⋮	⋮
n		5	2	7

- Run this gel with condition as 80 V with 1 h
- Observe with UV plate at 234 nm of wavelength to investigate the DNA band

For DNA amplification, 16S rRNA is used as the targeted position to multiply the number of DNA fragment. The procedure is shown (Scheme B-18).

Scheme B-18. Amplification step of LAB (Qiagen, Germany)

HotStar HiFidelity Polymerase Kit

- Prepare the polymerase kit as below

5x buffer	14 µl
10 µM Primer 27F (5’- AGA GTT TGA TCC TGG CTC AG -3’)	2.80 µl (0.4 µM final conc.)
10 µM Primer 1492R (5’- GGT TAC CTT GTT ACG ACT T -3’)	2.80 µl (0.4 µM final conc.)
2.5 units/µl HotStar polymerase	0.84 µl (2.1 units)
RNase-free water	46.56 µl
DNA template	3 µl
Total volume	**70 µl**

- Shortly spin the master mix
- Put in the T100 Thermocycler (Bio-rad, USA)
- Set the conditional programme below

Initial denaturation	95 °C	5 min
35x cycles		
Denaturation	94 °C	15 s
Annealing	46.4 °C	1 min
Extension	72 °C	1 min 45 s
Final Extension	72 °C	10 min

- Stored the PCR products at -20 °C for purification step

Scheme B-19. Purification step of LAB (BioFACT, South Korea)

PCR product checking

- Check the whole PCR product by gel electrophoresis
  Dye-colouring in 1% (w/v) agarose with 10,000-fold Gel Green nucleic acid gel stain (Vivantis, Malaysia)
- Load 70 µl of PCR products combined with 14 µl of 6x loading dye
- Run this gel with condition as 80 V with 1 hour
- Observe with UV plate at 234 nm of wavelength to investigate the DNA band
- Cut the promising band with blade and transfer to microtube

Purification step

- Add 600 µl of UB solution and incubate 65 °C for 10 min
- Add 200 µl of isopropanol
- Apply to spin column meticulously and centrifuge 7,000 rpm for 1 min
- Re-apply the waste to the spin column and centrifuge again
- Add 750 µl of WB and centrifuge 13,000 rpm for 30 s for twice washing
- Discard the waste out
- Re-centrifuge 13,000 rpm for 3 min to eliminate the liquid-drop out
- Combine the spin column to microtube
- Add 30 µl of 56 °C-incubated EB and incubate for 1 min
- Centrifuge 13,000 rpm for 2 min
- Store genomic DNA at -20 °C to send for sequencing step

Purified PCR products are checked with gel electrophoresis again before sending for DNA sequencing to calculate the DNA concentration. The DNA base information of sequencing step is encrypted (1st Base, Malaysia). After receiving the information, the sequences are applied in Nucleotide BLAST (NCBI 2018) to compare the most similarity of contestant strains and companion LAB before harvesting the most similarity LABs to establish a phylogenetic tree. Sequences are aligned with BioEdit version 7.6.2.1 (Hall 1999) and constructed by MEGA (Tamura et al. 2007). The tree is constructed to compare the contestant name and their relationships by Bootstrap method of phylogeny with a thousand replication tests in Jukes-Cantor substitution model in Neighbour-joining statistical method. An out-group is *Alloiococcus otitis* DSM7252^T (NR026088) to compare with their contestant strains.

10. Detection of antioxidant activity

10.1. Authentic samples for standard solution

10.1.1. 10 mM L-ascorbic acid standard solution

10 mM L-ascorbic acid stock standard solution is prepared following below, and dilution of authentic sample for DPPH and ABTS method is prepared in the Scheme B-20, while dilution for ORAC is provided (Scheme B-21).

L-ascorbic acid (MW = 176.19 g/mol)	8.81 mg
Deionised water	5 ml

Scheme B-20. Dilution of L-ascorbic acid (LAA) for DPPH and ABTS methods

1000 µM of L-ascorbic acid solution

- Add 1 ml of 10 mM L-ascorbic acid stock solution
- Dissolve with 9 ml of deionised water
- Make the prospective concentration in the following below

LAA standard solution (µM)	1000 µM LAA solution (µl)	Deionised water (µl)
500 (10 nmol)	500	500
400 (8 nmol)	400	600
300 (6 nmol)	300	700
200 (4 nmol)	200	800
100 (2 nmol)	100	900
25 (0.5 nmol)	25	975

Scheme B-21. Dilution of L-ascorbic acid for ORAC method

100 µM of L-ascorbic acid solution

- Add 1 ml of 10 mM L-ascorbic acid stock solution in 10% methanol
- Dissolve with 9 ml of 10% methanol to make 1000 µM concentration
- Dilute 10 folds with 10% methanol to perform 100 µM concentration
- Make the prospective concentration in the following below

LAA standard solution (µM)	100 µM LAA solution (µl)	10% methanol (µl)
100 (2.0 nmol)	1000	0
75 (1.5 nmol)	750	250
50 (1.0 nmol)	500	500
25 (0.5 nmol)	250	750

10.1.2. 10 mM Trolox standard solution

10 mM trolox stock standard solution is prepared following below. Trolox standard solution for DPPH and ABTS methods is used the range (Scheme B-22). For ORAC method, trolox standard solution range is performed (Scheme B-23).

(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) 10.01 mg

Methanol 4 ml

Scheme B-22. Dilution of trolox standard solution for DPPH and ABTS methods

1000 µM of trolox solution

- Add 1 ml of 10 mM trolox stock solution
- Dissolve with 9 ml of deionised water (10% methanol as final solvent concentration)
- Make the prospective concentration in the following below

Trolox standard solution (µM)	1000 µM trolox solution (µl)	10% methanol (µl)
500 (10 nmol)	500	500
400 (8 nmol)	400	600
300 (6 nmol)	300	700
200 (4 nmol)	200	800
100 (2 nmol)	100	900
25 (0.5 nmol)	25	975

Scheme B-23. Dilution of trolox standard solution for ORAC method

100 µM of trolox solution

- Add 1 ml of 10 mM trolox stock solution
- Dissolve with 9 ml of methanol to make 1000 µM trolox solution in methanol
- Dilute 10 folds with pure methanol to make 100 µM trolox solution
- Make the prospective concentration in the following below

Trolox standard solution (µM)	100 µM trolox solution (µl)	Methanol (µl)
40 (0.8 nmol)	400	600
30 (0.6 nmol)	300	700
20 (0.4 nmol)	200	800
10 (0.2 nmol)	100	900

10.1.3. 10 mM gallic acid standard solution

10 mM gallic acid stock standard solution is prepared following below. Standard solution range for DPPH and ABTS methods is performed following (Scheme B-24), while solution for ORAC method is performed (Scheme B-25).

Gallic acid (MW = 170.12 g/mol)	8.51 mg
Deionised water	5 ml

Scheme B-24. Dilution of gallic acid standard solution for DPPH and ABTS methods

1000 µM of gallic acid solution

- Add 1 ml of 10 mM gallic acid stock solution
- Dissolve with 9 ml of deionised water to make 1000 µM concentration
- Make the prospective concentration in the following below

Gallic acid standard solution (µM)	**1000 µM gallic acid solution (µl)**	**Deionised water (µl)**
200 (4 nmol)	200	800
150 (3 nmol)	150	850
100 (2 nmol)	100	900
50 (1 nmol)	50	950
25 (0.5 nmol)	25	975
10 (0.2 nmol)	10	990

Scheme B-25. Dilution of gallic acid standard solution for ORAC method

100 µM of gallic acid solution

- Add 1 ml of 10 mM gallic acid stock solution in 10% methanol
- Dissolve with 9 ml of 10% methanol to make 1000 µM concentration
- Dilute 10 folds with 10% methanol to make 100 µM concentration
- Make the prospective concentration in the following below

Gallic acid standard solution (µM)	**100 µM gallic acid solution (µl)**	**10% methanol (µl)**
40 (0.8 nmol)	400	600
30 (0.6 nmol)	300	700
20 (0.4 nmol)	200	800
10 (0.2 nmol)	100	900

10.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) method

10.2.1. Reagent for DPPH method

10.2.1.1. 107.14 µM DPPH solution

DPPH (MW = 394.32 g/mol)	2.11 mg
Methanol	50 ml

After finished preparation, this solution is kept by aluminium foil protection and should be prepared before experimental working.

10.2.2. Procedure for DPPH method

Radical scavenging activity (DPPH) testing is modified (Banerjee et al. 2005) (Scheme B-26).

Scheme B-26. Step of radical scavenging activity testing

Put samples into each microplate well in the following below

Negative control (Blank)	Positive control	Sample	Standard
20 µl suitable solvent[a]	20 µl suitable solvent[a]	20 µl sample	20 µl standard solution[b]
280 µl methanol	280 µl DPPH solution	280 µl DPPH solution	280 µl DPPH solution

Incubate 30 min in dark room condition

Examine with spectrophotometry technique at OD_{517}

Note: [a] The suitable solvent for positive and negative controls is depended on solution. For L-ascorbic acid and gallic acid standard solution, deionised water is used for those controls. For trolox standard solution, 10% methanol is used for those controls. For sample, it is depended on solvent of sample extraction.

[b] Standard solution used for DPPH method testing is composed of trolox, L-ascorbic acid, and gallic acid.

After the test had finished, the percentage of radical scavenging activity (% RSA) is calculated in the formula below, and antioxidant activity is also calculated to report in term of trolox equivalent (µmol TE/ml sample).

$$\% \text{ RSA} = \frac{A_p - A_s}{A_p} \times 100\%$$

When A_p = absorbance of completely scavenging activity (positive control)

A_s = absorbance of antioxidant-scavenging activity (sample)

IC_{50} is calculated from the equation of standard curve between % radical scavenging activity (% RSA) and the authentic sample concentration in µM in which 50% RSA is substituted in the equation to find the half-maximum concentration.

For the regression curve of DPPH for each authentic sample, the curve illustrates between % RSA, and each authentic sample concentration (µM) is shown (Figure 4-15), while the curve illustrates between absorbance OD_{517}, and each authentic sample mass (nmol) is previewed (Figure B-7 and Figure B-8).

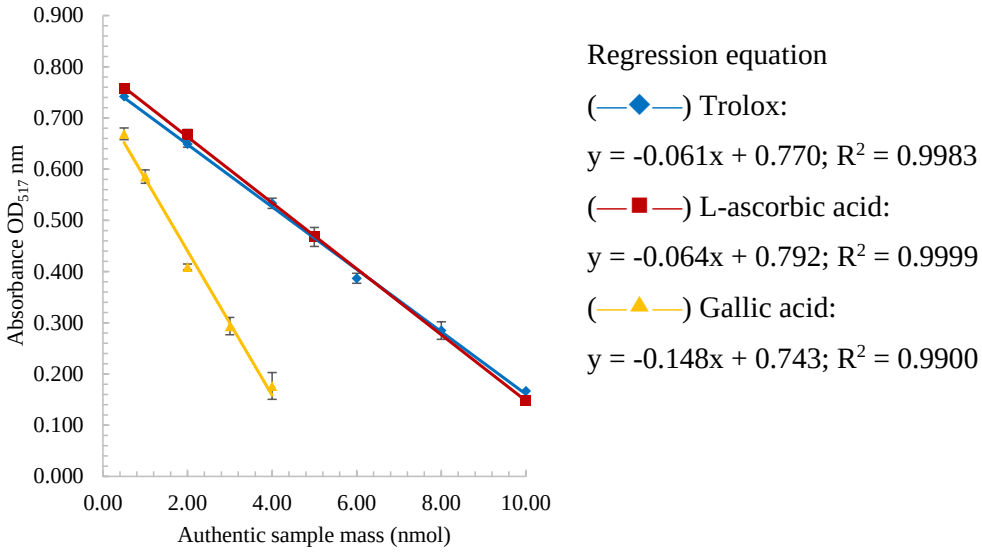


Figure B-7. Regression curve of DPPH between absorbance OD_{517} and each authentic sample mass (nmol)

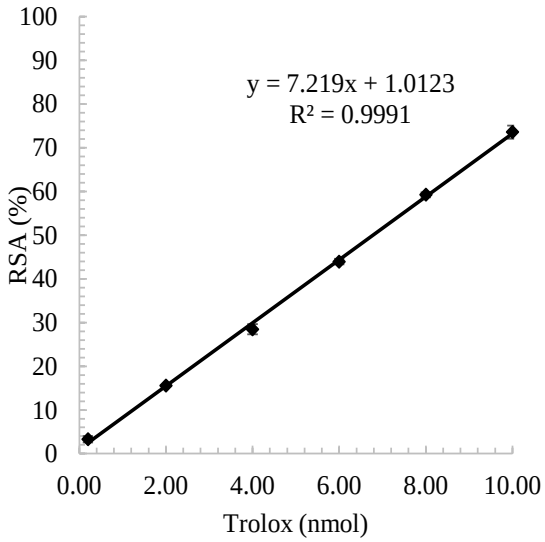


Figure B-8. Regression equation of DPPH between RSA (%) and trolox mass (nmol) with regression equation as y = 7.219x + 1.0123; R^2 = 0.9991

10.3. 2,2'-azinobiz (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

10.3.1. Reagent for ABTS method

10.3.1.1. 107.14 µM ABTS solution

Solution A: 5 mM ABTS stock solution

Solution A-1: 7 mM ABTS

ABTS (MW = 514.62 g/mol)	18.01 mg
Deionised water	5 ml

Solution A-2: 8.75 mM potassium peroxosulfate ($K_2S_2O_8$)

$K_2S_2O_8$ (MW = 270.21 g/mole)	4.73 mg
Deionised water	2 ml

Solution B: 375 µM ABTS working solution

ABTS stock solution	1.50 ml
Deionised water	18.50 ml

Solution C: 107.14 µM ABTS working solution

375 µM ABTS working solution	20 ml
Deionised water	50 ml

To prepare the ABTS solution, 5 ml of solution A-1 are mixed with 2 ml of solution A-2. The final mole ratio of those solutions is 1: 0.5 at 5 mM ABTS: 2.5 mM $K_2S_2O_8$. After mixing the solution, the colour of solution is turned from greenish tea colour to dark greenish-blue colour with complete reaction at least 16 h. Before testing, solution B and C are respectively diluted with deionised water to make the final concentration at 107.14 µM.

10.3.2. Procedure for ABTS method

Trolox is one of the popular standard antioxidative agents for ABTS experiment. The procedure for ABTS is modified from (Re et al. 1999) that have the description (Scheme B-27).

Scheme B-27. Procedure of ABTS method

Put analytes into microplate well in the following

Negative control (Blank)	**Positive control**	**Sample**	**Standard**
20 µl suitable solvent[a]	20 µl suitable solvent[a]	20 µl sample	20 µl standard solution[b]
280 µl deionised water	280 µl ABTS solution	280 µl ABTS solution	280 µl ABTS solution

↓ incubate for 3 min at room temperature

↓ Examine with spectrophotometry at OD_{734}

Note: [a] The suitable solvent for positive and negative control is depended on solution. For L-ascorbic acid and gallic acid standard solution, deionised water is used for those controls. For trolox standard solution, 10% methanol is used of those controls. For sample, it is depended on solvent of sample extraction.

[b] Standard solution used for ABTS method testing is composed of trolox, L-ascorbic acid, and gallic acid.

The percentage of ABTS inhibition is calculated from the equation below, and antioxidant activity is reported in term of trolox equivalent (µmol TE/ml sample)

$$\% \text{ inhibition of ABTS} = \frac{A_p - A_s}{A_p} \times 100\%$$

When A_p = absorbance of completely oxidised oxidation (positive control)

A_s = absorbance of antioxidant-oxidised oxidation (sample)

IC_{50} is calculated from the equation of standard curve between % inhibition and authentic sample concentrations in µM in which 50 % inhibition is substituted in the equation to find the half-maximum concentration.

For the regression curve of ABTS for each authentic sample, the curve illustrated between % inhibition, and each authentic sample concentration (µM) is shown (Figure 4-15), while the curve illustrates between absorbance OD_{734}, and each authentic sample mass (nmol) is presented (Figure B-9 and Figure B-10).

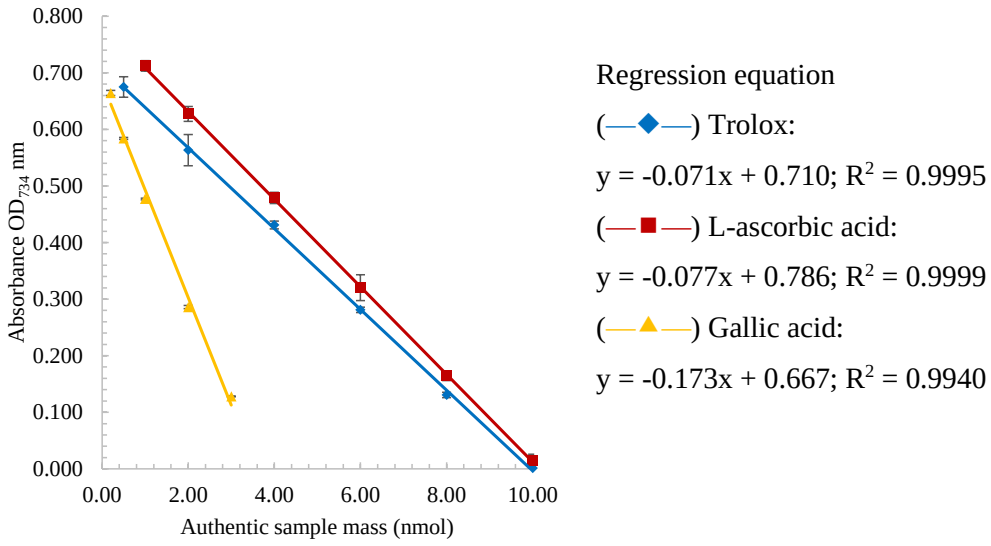


Figure B-9. Regression curve of ABTS between absorbance OD_{734} and each authentic sample mass (nmol)

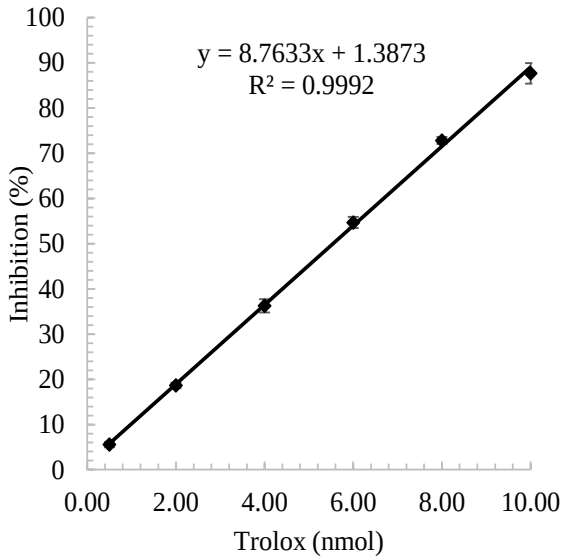


Figure B-10. Regression equation of ABTS between inhibition (%) and trolox mass (nmol) with regression equation as y = 8.7633x + 1.3873; R^2 = 0.9992

10.4. Oxygen radical antioxidant capacity (ORAC) method

10.4.1. Reagent for ORAC method

10.4.1.1. Phosphate buffer solution pH 7.4

Solution A: 150 mM phosphate buffer stock solution pH 7.4

Solution A-1: Di-potassium hydrogen phosphate (K_2HPO_4)

K_2HPO_4 (MW = 174.18 g/mol)	2.6127 g
Deionised water	100 ml

Solution A-2: Potassium di-hydrogen phosphate (KH_2PO_4)

KH_2PO_4 (MW = 136.08 g/mol)	0.5103 g
Deionised water	25 ml

Solution B: 75 mM phosphate buffer working solution pH 7.4

150 mM phosphate buffer stock solution pH 7.4	10 ml
Deionised water	10 ml

After finishing to prepare both solution A-1 and A-2 in volumetric flask, buffer solution is established by mixing both solutions altogether and adjusted pH to 7.4 by pH meter to 100 ml as the final volume. This solution is kept in 4 °C until the using time. Working solution (solution B) must be prepared before experimental beginning from buffer stock solution and discarded after finishing experiment.

10.4.1.2. Fluorescein solution

Solution A: 1.2 mM fluorescein stock solution 1	
Fluorescein sodium salt (MW = 376.27 g/mol)	0.04515 g
75 mM phosphate buffer pH 7.4	100 ml
Solution B: 6 µM fluorescein stock solution 2	
1.2 mM fluorescein stock solution 1	100 µl
75 mM phosphate buffer pH 7.4	19.9 ml
Solution C: 150 nM fluorescein working solution	
6 µM fluorescein stock solution 2	100 µl
75 mM phosphate buffer pH 7.4	3.9 ml

After mixing 100 ml of total volume of solution A, this solution can keep at 4 °C with aluminium foil protection. For solution B, after mixing 20 ml of total volume, this solution can keep at 4 °C with aluminium foil protection. Finally, for solution C, after mixing 4 ml of total volume, this solution should be prepared on the experimental day and used until empty for experimental assessment. During the experiment, this solution should be protected away from ambient light by aluminium foil protection.

10.4.1.3. 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) radical solution for ORAC

AAPH (MW = 271.19 g/mol)	101.70 mg
75 mM phosphate buffer pH 7.4 at 37 °C	2 ml

This solution should be immediately prepared by adding the warming phosphate buffer at 37 °C and heated by a heat box around 30 min to activate the radical molecules and discarded after finishing experiment.

10.4.2. Procedure for ORAC method

Oxygen radical antioxidant capacity (ORAC) method is modified (Ghiselli et al. 1995; Glazer 1990) to be more suitable for this experiment. Step for performing ORAC will be described (Scheme B-28).

Scheme B-28. Procedure of ORAC method

Put analytes into microplate black well in the following

Blank	**Sample**	**Standard**
20 µl suitable solvent[a]	20 µl sample	20 µl standard solution[b]

- Cover the used well with Aeraseal film
- Evaporated with vacuum pump in glass desiccator for 15 min or fully evaporate
- 20 µl 10% methanol
- 80 µl 75 mM phosphate buffer solution
- 80 µl 150 mM fluorescein solution
- Incubate at 37 °C for 30 min
- 20 µl 187.5 mM AAPH solution[c]
- Examine with fluorescence microplate reader at 480 nm of excited wavelength and 520 nm to detect emitted wavelength

Note: [a] Suitable solvent is depended on used solution. For trolox standard solution, 10% methanol was used and for sample, it is depended on solvent of sample extraction.

[b] Trolox standard solution is only used as the main authentic sample. L-ascorbic acid and gallic acid are supported for the other authentic samples for this testing to compare and find ORAC score.

[c] Adding AAPH solution activated by warming phosphate buffer could be meticulously done. The radical molecules were generated after adding and dissolving the warming phosphate buffer. The radical-inhibited reaction is immediately started after adding AAPH solution to the reaction.

After finishing the measurement, raw data of every concentration is combined altogether to make a curve. Firstly, raw data of fluorescence intensity (FI) is calculated to base on portion one thanks to re-adjustment of in-equality of FI at the origin point. Secondly, area under the curve (AUC) is found from the area integration between fluorescence intensity (FI) of portion one and time in second unit. Finally, net AUC is calculated from AUC of samples cutting by AUC of blank. Curves between AUC and time are illustrated (Figure B-11). For regression curve between net AUC and authentic sample mass (nmol) is presented (Figure B-12).

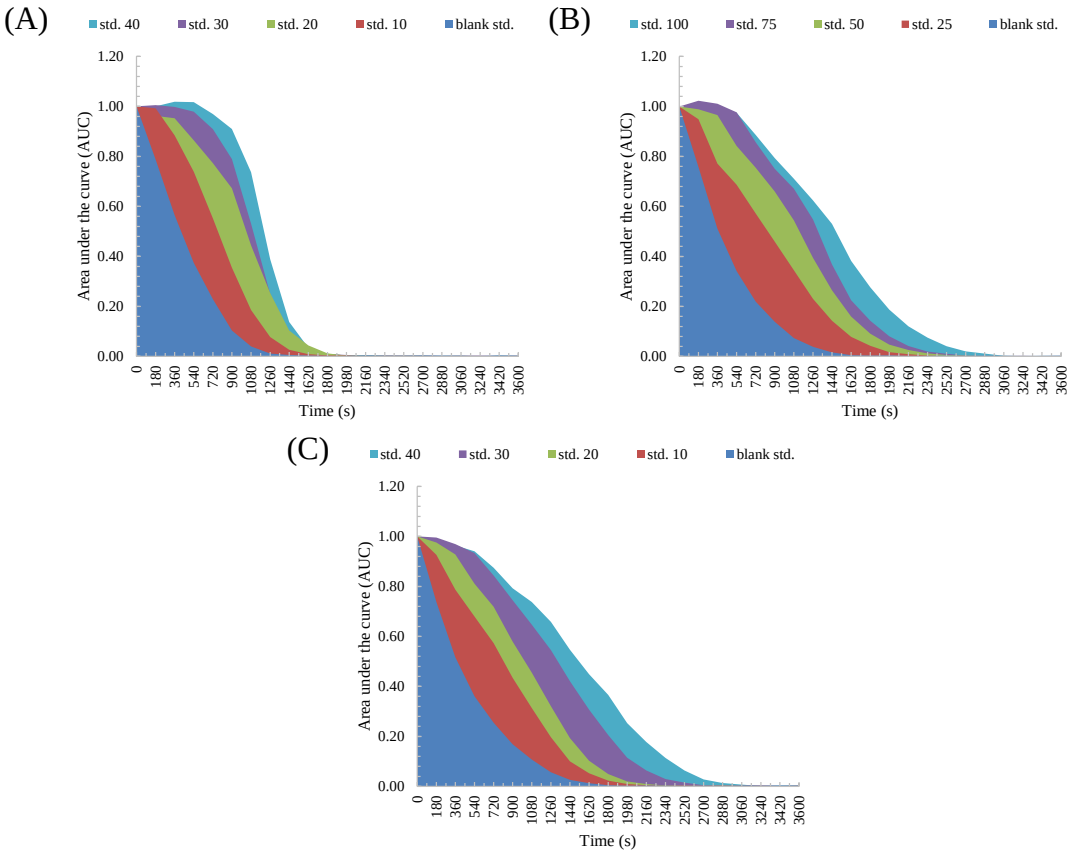


Figure B-11. ORAC curves of each authentic sample between AUC and time with (A) trolox, (B) L-ascorbic acid, and (C) gallic acid

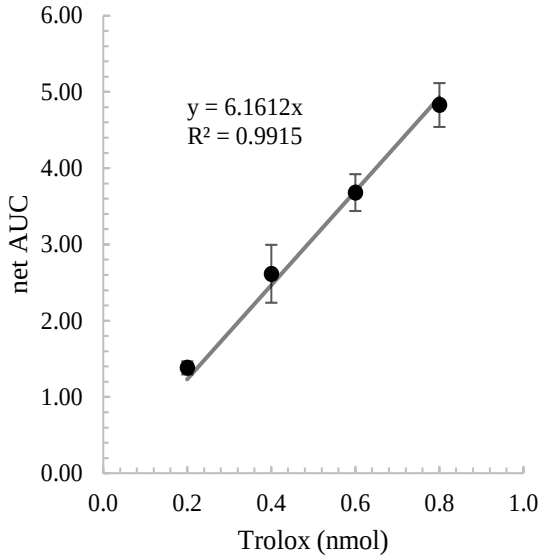


Figure B-12. Regression equation of ORAC between net AUC and trolox mass (nmol) with regression equation as y = 6.1612x with R^2 = 0.9915

11. Determination of total phenolic content (TPC)

11.1. Reagent for TPC method

11.1.1. 0.2 N Folin-Ciocalteu reagent

2 N Folin-Ciocalteu's phenol reagent	2 ml
Deionised water	18 ml

This solution is freshly prepared and kept away from ambient light by covering by aluminium foil.

11.1.2. 75 g/L sodium carbonate (Na_2CO_3)

Sodium carbonate (Na_2CO_3)	7.50 g
Deionised water	100 ml

This solution can place in ambient room after finishing preparation.

11.2. Procedure for TPC method

TPC was modified (Zhao et al. 2014) to evaluate the content of phenolic compounds (Scheme B-29).

Scheme B-29. Method of total phenolic content (TPC)

Put analytes into a microplate well in the following

Blank	**Sample**	**Standard**
20 µl suitable solvent[a]	20 µl sample	20 µl gallic acid standard solution[b]

- 200 µl of deionised water
- 20 µl 0.2 N Folin-Ciocalteu reagent solution
- Room temperature incubation for 5 min
- 60 µl of 75 g/L Na_2CO_3 solution
- Room temperature incubation for 30 min
- Examination with spectrophotometry method at OD_{765}

Note: [a] The suitable solvent depends on solution usage. For gallic acid standard solution, deionised water is used, and sample depends on extracted solvent.

[b] Gallic acid standard solution was only used for TPC testing.

After finishing examination, the information is evaluated with total phenolic contents in gallic acid equivalents (µmol GE/ml sample).

$$\text{Total phenolic content} = \frac{Gallic\ acid\ standard\ (\mu mol)}{Sample\ (ml)}$$

11.3. 10 mM gallic acid standard solution

10 mM of gallic acid stock standard solution is prepared following below. Standard solution range for TPC method is performed following in the Scheme B-30.

Gallic acid (MW = 170.12 g/mol)	8.51 mg
Deionised water	5 ml

Scheme B-30. Dilution of gallic acid standard solution for TPC

1000 µM of gallic acid solution

- Add 1 ml of 10 mM gallic acid stock solution
- Dissolve with 9 ml of deionised water
- Make the prospective concentration in the following below

Gallic acid standard solution (µM)	**1000 µM gallic acid solution (µl)**	**Deionised water (µl)**
750	750	250
500	500	500
250	250	750
100	100	900
50	50	950
25	25	975

Total phenolic content regression curve and equation illustrate in the Figure B-13.

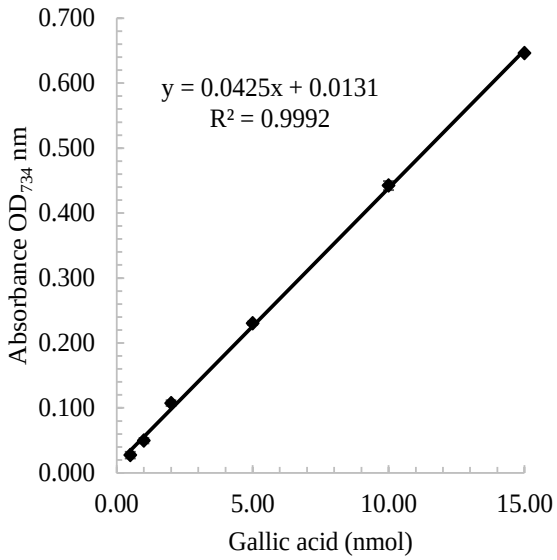


Figure B-13. Regression curve of TPC between absorbance OD_{734} and gallic acid mass (nmol) as standard authentic sample with regression equation as $y = 0.0425x - 0.0131$; $R^2 = 0.9992$

12. Acid content

12.1. Chemical preparation

Acid titration is referred the method (AOAC 2005) with the following.

12.1.1. Carbon dioxide-eliminated distilled water

Distilled water is prepared in Duran's bottle by boiling until heating to eliminate carbon dioxide out. After finishing boil, cap of bottle is tightly closed to protect air to dissolve in the water. This water could be leaved until cold with room temperature before using.

12.1.2. 0.1 M sodium hydroxide (NaOH)

Sodium hydroxide (MW = 40 g/mol)	1.00 g
Carbon dioxide-eliminated distilled water	250 ml

Sodium hydroxide is weighed and put in the volumetric flask to dissolve with that distilled water until 250 ml.

12.1.3. Potassium hydrogen phthalate ($KHC_8H_4O_4$)

This chemical is prepared by weigh around 1.00 g to put in aluminium cup which is baked at 105 °C for 2 h before. After weighing the chemical, this chemical was baked with 105 °C for 2 h to eliminate the moisture out. Then this chemical is removed out and kept in a desiccator until testing date.

12.1.4. 10 mg/ml phenolphthalein reagent

Phenolphthalein ($C_{20}H_{14}O_4$)	0.10 g
Absolute ethanol	10 ml

Phenolphthalein chemical is weighed and dissolved with absolute ethanol. Then NaOH solution is dropped until the colour turned to light-pinkish colour. One-fold distilled water is added to dilute.

12.2. Acid titration procedure

12.2.1. Testing the unknown sodium hydroxide solution

Before staring acid titration, concentration of sodium hydroxide solution could be known. Procedure to find the concentration is followed in the Scheme B-31.

Scheme B-31. Finding unknown sodium hydroxide solution

Unknown NaOH solution

- Weigh around 0.30 g of baked-$KHC_8H_4O_4$ to put in 25-ml-sizing of Erlenmeyer flask and note the weight for stoichiometric calculation
- Add 50 ml of carbon dioxide-eliminated distilled water and dissolve
- Drop 3 drops of phenolphthalein reagent and mix
- Titrate with unknown NaOH solution in burette
- Note the volume of NaOH in burette after applying of light-pinkish colour
- Calculate to find concentration of NaOH solution

$$N = \frac{g \times 1000}{204.229 \times V}$$

When g = weight of potassium hydrogen phthalate (g)

204.229 = molecular weight of $KHC_8H_4O_4$ (g/mol)

V = titrated volume of sodium hydroxide solution (ml)

N = concentration of sodium hydroxide solution (M)

12.2.2. Acid titration procedure

To find the acid contents from samples, acid titration is one of various methods to respond. The procedure is following in the Scheme B-32.

Scheme B-32. Acid titration

Samples

- Place 1 ml of sample in Erlenmeyer flask
- Add 25 ml of carbon dioxide-eliminated distilled water
- Drop 3 drops of phenolphthalein reagent and mix
- Titrate with known NaOH solution in burette
- Note the used volume of NaOH in burette after applying of light-pinkish colour
- Calculate to find acid titration with the following formula

$$\%\,Acid = \frac{N \times V \times 90.08 \times 100}{1000 \times 1}$$

When 90.08 = molecular weight of lactic acid (g/mol)

N = concentration of sodium hydroxide solution (M)

V = titrated volume of sodium hydroxide (ml)

% Acid = acid content (% g/100 ml)

13. Whey extraction from milk for ACE inhibitor and GABA determination

13.1. Reagent for extraction

13.1.1. 1 M hydrochloric acid (HCl) solution for whey extraction

37% (w/w) fuming hydrochloric acid (MW = 36.5 g/mol, ρ = 1.19 g/ml) 4.14 ml

50 ml of distilled water

Concentrated hydrochloric acid is pipetted to volumetric flask and diluted with distilled water up to 50 ml in that volumetric flask.

13.2. Extraction procedure

Procedure of whey extraction for ACE inhibitor testing and GABA determination is referred by (Abubakr et al. 2012) showing in the Scheme B-33.

Scheme B-33. Whey extraction for ACE-I testing

Milk sample

- Harvest 1.2 ml of fermented milk in each treatment
- Add 50 µl of 1 M HCl to suitable volume for pH around 4.6
- Mix this milk with vortex
- Boil at 80 °C for 10 min
- Centrifuge at 17,000 × *g* for 10 min to separate casein-agglutinating part
- Remove whey supernatant out to another microtube
- Store this whey at -20 °C until testing date

14. Efficiency of ACE inhibitor

14.1. Reagent for testing of ACE inhibitor

14.1.1. 0.1 M HEPES Buffer pH 8.3

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; MW = 238.30 g/mol)	1.19 g
Deionised water	30 ml

After HEPES is fully dissolved by deionised water, pH is adjusted by 10 M of NaOH for pH adjustment to pH at 8.3. In the next, the following chemicals are added, respectively.

Sodium chloride (NaCl; MW = 58.5 g/mol)	0.88 g
Triton X-100	5 µl
Deionised water	20 ml

The above chemicals are added and dissolved. This solution is removed to 50-ml-sizing of volumetric flask. Deionised water is added until 50 ml fulfilment. Stream-sterilisation needs to sterilise this solution at 121 °C for 15 min.

14.1.2. Storage buffer: 0.01 M phosphate buffer pH 7.0 with 0.5 M NaCl

Solution A: 0.01 M K_2HPO_4

di-Potassium hydrogen phosphate (K_2HPO_4; MW = 174.18 g/mol)	174.20 mg
deionised water	100 ml

Solution B: 0.01 M KH_2PO_4

Potassium di-hydrogen phosphate (KH_2PO_4; MW = 136.09 g/mol)	136.10 mg
deionised water	100 ml

Solution A and B are mixed altogether and measured with pH meter to establish buffer solution at pH 7.0

Solution C: 0.01 M phosphate buffer pH 7.0 supplemented with 0.5 M NaCl

Sodium chloride (NaCl)	2.93 g
0.01 M phosphate buffer pH 7.0 from the above	100 ml

After mixing solution C altogether, stream-sterilisation needs to perform at 121 °C for 15 min.

14.1.3. Stock enzyme solution: 2000 mU/ml ACE enzyme solution

ACE enzyme (A6778-0.1UN; 100 mU; 2.0 U/mg protein; sigma Aldrich, USA) is purchased. After calculation, protein content of this enzyme in purchased vial is 0.05 mg protein. With the company recommendation to storage this enzyme, the recommended enzyme concentration is 1 mg/ml in storage buffer. Procedure can do in the Scheme B-34.

Scheme B-34. Storage enzyme in storage buffer

1 mg/ml ACE enzyme in storage buffer

- Add 50 µl of storage buffer in purchased enzyme vial
- Mix by thoroughly vortex
- Be aliquot for 10 µl of enzyme solution to 300 µl of microtube
- Store at -20 °C following the company recommendation with guarantee life for several months.

14.1.4. Working enzyme solution: 20 mU/ml ACE enzyme solution

Enzyme stock solution	10 µl
Sterilised 0.1 M HEPES buffer pH 8.3	990 µl

One stock enzyme solution is throwed and replaced to 1.5 ml sizing of microtube. HEPES buffer is added to dilute this enzyme for working enzyme solution.

14.1.5. 8 mM ACE substrate (Hippuryl-histidyl-leucine)

ACE substrate	3.44 mg
(MW = 429.47 g/mol; H1635-25MG; sigma, the United States)	
Dimethyl sulfoxide (DMSO)	200 µl
Sterilised deionised water	800 µl

These ingredients are mixed altogether and made 1 ml as final volume.

14.1.6. 0.1 M sodium hydroxide

Sodium hydroxide (MW = 40 g/mol; NaOH)	0.20 g
Deionised water	50 ml

This ingredient is dissolved with deionised water in volumetric flask.

14.1.7. 10 mg/ml o-phthaldialdehyde (OPA) solution

O-phthaldialdehyde (MW = 134.13 g/mol; OPA)	0.02 g
Methanol	2 ml

This ingredient is dissolved with methanol in 2 ml of volume.

14.1.8. 0.1 M hydrochloric acid (HCl) solution for ACE inhibitor testing

37% (w/w) fuming hydrochloric acid (MW = 36.5 g/mol, ρ = 1.19 g/ml) 414 µl
50 ml of deionised water

Concentrated hydrochloric acid is pipetted to volumetric flask and diluted with deionised water up to 50 ml in that volumetric flask.

14.2. Procedure of testing of ACE inhibitor

14.2.1. Procedure of efficiency testing of ACE inhibitor

This procedure is referred (Nakano et al. 2006) using fluorescence technique detection in a 96-well microtiter black well plate. Procedure is followed in the Scheme B-35.

Scheme B-35. Step of ACE inhibitor testing

ACE inhibitor testing

- Add 25 µl of HEPES buffer

Sample	Positive control	Negative control
5 µl sample	5 µl HEPES buffer	5 µl HEPES buffer

- Add 10 µl of 20 mU/ml ACE enzyme solution (working solution)
- Incubate at 37 °C for 5 min
- Add 10 µl of ACE substrate
- Incubate at 37 °C for 30 min
- Add 40 µl of 0.1 M NaOH solution and 20 µl of OPA solution
- Incubate at room temperature for 30 min
- Add 100 µl of 0.1 M hydrochloric acid solution
- Incubate at 37 °C for 30 min
- Measure dependent His-Leu-OPA with fluorescence spectrophotometric detection with 355 nm and 460 nm of excited and emitted wavelength

After finishing for measurement, calculation to find % inhibition needs with the formula below.

$$\%\ inhibition = \frac{P - S}{P} \times 100\%$$

When P = emission of complete fluorescence intensity (positive control)

S = emission of enzyme inhibition fluorescence intensity (sample)

15. Extraction of antioxidative compounds in dairy products: Methanol/Acetonitrile method (MeOH/ACN)

15.1. Chemical preparation

15.1.1. Methanol: dIH_2O (1:1) extracted solution

Methanol	10 ml
Deionised water	10 ml

This mixture can prepare before extraction date and store in room temperature for long time.

15.1.2. Carrez I solution

Potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$)	1.50 g
Deionised water	10 ml

This solution after finishing the preparation could be covered with aluminium foil to protect away from the light and store in 4 °C refrigerator.

15.1.3. Carrez II solution

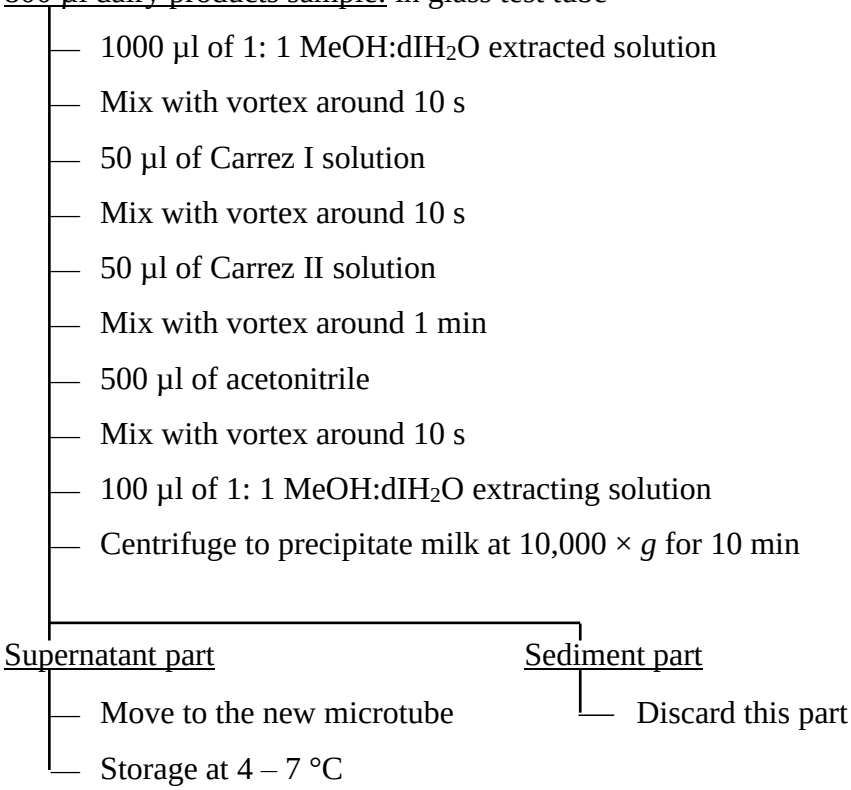
Zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	3.00 g
Deionised water	10 ml

This solution after finishing the preparation could be covered with aluminium foil to protect away from the light and store in 4 °C refrigerator.

15.2. Extracted procedure

This method is referred and adapted from (Vázquez et al. 2015) to extract antioxidative compounds especially from milk and dairy products. The procedure is clarified in the Scheme B-36.

Scheme B-36. Dairy products extraction by methanol/acetonitrile method



After finishing preparation, total volume of this extraction is 2,500 µl. Then the dilution of this extraction was 3.125 folds. This extraction should be kept at -25 °C (not ice-clotting) until the testing date. Ice-clotting can cause the degradation of radical compound. Total volume is noted to calculate. The antioxidant activity and total phenolic content are determined as described procedure above (Topic 10 and 11, Appendix B).

APPENDIX C

Additional information of LAB molecular identification

1. ENM104

LOCUS MN915130 1445 bp DNA linear BCT 09-JAN-2020
DEFINITION *Pediococcus pentosaceus* strain ENM104 16S ribosomal RNA gene, partial sequence.
ACCESSION MN915130
VERSION MN915130
KEYWORDS .
SOURCE *Pediococcus pentosaceus*
ORGANISM *Pediococcus pentosaceus*
Bacteria; *Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Pediococcus*.
REFERENCE 1 (bases 1 to 1445)
AUTHORS Jitpakdee,J.
TITLE Isolation and selection of cholesterol-lowering *Pediococcus pentosaceus* ENM104
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1445)
AUTHORS Jitpakdee,J.
TITLE Direct Submission
JOURNAL Submitted (09-JAN-2020) Microbiology, Prince of Songkla University, 15 Karnjanavanich Road, Hat Yai, Songkhla 90110, Thailand
COMMENT Sequences were screened for chimeras by the submitter using BioEdit 7.0.5.
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2. SPS109

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DEFINITION *Lactobacillus plantarum* strain SPS109 16S ribosomal RNA gene, partial sequence.
ACCESSION MN915131
VERSION MN915131
KEYWORDS .
SOURCE *Lactobacillus plantarum*
ORGANISM *Lactobacillus plantarum*
Bacteria; *Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus*.
REFERENCE 1 (bases 1 to 1449)
AUTHORS Jitpakdee,J.
TITLE Isolation and selection of gamma-aminobutyric acid-producing Lactobacillus plantarum SPS109
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1449)
AUTHORS Jitpakdee,J.
TITLE Direct Submission
JOURNAL Submitted (09-JAN-2020) Microbiology, Prince of Songkla University, 22 Soi 24 Khlongtoei, Khohong, Hat Yai, Songkhla 90110, Thailand
COMMENT Sequences were screened for chimeras by the submitter using BioEdit 7.0.5.

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3. ENM203

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5. NNM205

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APPENDIX D

Calculation of kinetic growth

From the Figure 4-7, this curve describes bacterial growth characteristics to predict doubling time of both bacterial growths. From the theory that bacterial turbidity is doubled, bacterial cell numbers are also doubled. With the exponential pattern of bacterial growth, Figure D-1 displays the growth in term of logarithmic absorbance OD_{660} by time in h.

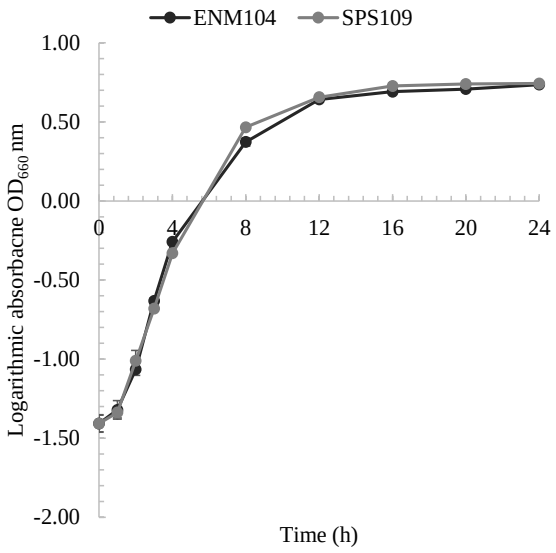

Figure D-1. Bacterial growth in term of logarithmic absorbance OD_{660} by time in h of both contestant LAB strains including ENM104 (●) and SPS109 (●)

Range of consideration for doubling time estimation is selected the greatest growth in exponential phase due to the maximum number of cell division. Then the time 1st to 4th h are selected to make the regression curve showing in the Figure D-2.

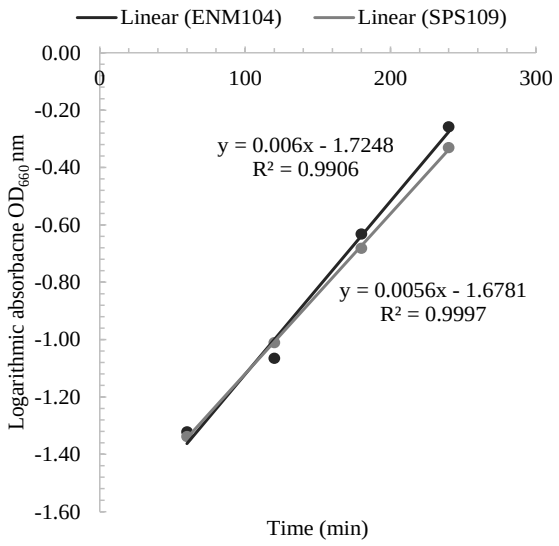

Figure D-2. Regression curve of both contestant LAB strains including ENM104 (●) with the regression equation as y = 0.006x – 1.7248; R^2 = 0.9906 and SPS109 (●) with the regression equation as y = 0.0056 x – 1.6781; R^2 = 0.9997

Theory of cell division rate is from the comparison between cell increasing by time change. Then the relationship equation is shown in the below.

$\frac{\Delta N}{\Delta t} \sim N$ when N = rate of cell division

ΔN = cell increase

Δt = time change

Then $\frac{dN}{dt} = \mu N$ when μ = constant number

Switch $\frac{1}{N} \cdot dN = \mu dt$

Integration $\int \frac{1}{N} \cdot dN = \mu \int dt$ from integration rule

$\int \frac{1}{N} \cdot dN = \ln N$ and $\int dt = t$

Then $\ln N = \mu t + c$ **… equation 1**

When c = constant number of integrations

When t = 0 putting in equation 1;

Then $ln\,N_0 = c$ **… equation 2**

Put equation 2 in equation 1

$$ln\,N = \mu t + ln\,N_0$$

Switch $ln\,N - ln\,N_0 = \mu t$ **… equation 3**

Then $ln\frac{N}{N_0} = \mu t$ **… equation 4**

From $ln\,A - ln\,B = ln\frac{A}{B}$

From the equation 3, cell increases based on turbidity increase

Then $ln\,OD - ln\,OD_0 = \mu t$ **… equation 5**

when $N = OD$ and $N_0 = OD_0$

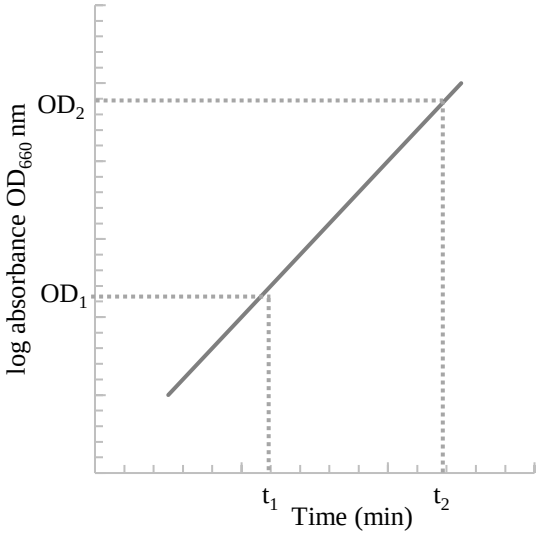

Figure D-3. Relationship of turbidity and time

Cell numbers depend on cell turbidity (Figure D-3). Then doubling time is from doubling of cell turbidity from equation 5.

Then $ln\,OD_2 - ln\,OD_0 = \mu t_2$ **… equation 6**

And $ln\,OD_1 - ln\,OD_0 = \mu t_1$ **… equation 7**

Equation 6 – equation 7;

$$(\ln OD_2 - \ln OD_0) - (\ln OD_1 - \ln OD_0) = \mu t_2 - \mu t_1$$

Then $\ln OD_2 - \ln OD_1 = \mu(t_2 - t_1)$

Switch $\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$

$\mu = \frac{\ln \frac{OD_2}{OD_1}}{t_2 - t_1}$ From $\ln A - \ln B = \ln \frac{A}{B}$

$\mu = \frac{\log_e \frac{OD_2}{OD_1}}{t_2 - t_1}$ From $\ln A = \log_e A$

$\mu = \frac{(\log_e 10)(\log_{10} \frac{OD_2}{OD_1})}{t_2 - t_1}$ From $\log_a B = (\log_a C)(\log_c B)$

$\mu = \frac{(\ln 10)(\log_{10} \frac{OD_2}{OD_1})}{t_2 - t_1}$ From $\log_e A = \ln A$

$\mu = \frac{(\ln 10)(\log OD_2 - \log OD_1)}{t_2 - t_1}$ **… equation 8**

From $\log_{10} A = \log A$ and $\ln A - \ln B = \ln \frac{A}{B}$

From the Figure D-2, time of regression curve stands in 60 – 240 min

ENM104	**SPS109**
$y = 0.0060x - 1.7249$; $R^2 = 0.9906$	$y = 0.0056x - 1.6781$; $R^2 = 0.9997$
$\log OD = 0.0060t - 1.7249$ **... equation 9**	$\log OD = 0.0056t - 1.6781$ **... equation 9**
When x = t = time (min) and y = log OD = log OD_{660}	

Put equation 9 in each regression equation and obvious turbidity at 0.2 and 0.4 were selected as candidates.

$0.4 = 0.0060t_2 - 1.7249$	$0.4 = 0.0056t_2 - 1.6781$
When $\log OD_2 = 0.4$	*When $\log OD_2 = 0.4$*
And $t_2 = 351.83$ min (5.86 h)	*And $t_2 = 372.01$ min (6.20 h)*
$0.2 = 0.0060t_2 - 1.7249$	$0.2 = 0.0056t_2 - 1.6781$
When $\log OD_1 = 0.2$	When $\log OD_1 = 0.2$
And $t_1 = 318.71$ min *(5.31 h)*	And $t_1 = 336.21$ min *(5.60 h)*

Put log OD_2, t_2, log OD_1 and t_1 in equation 8

$\mu = \frac{(ln10)(0.4-0.2))}{351.83-318.71}$ $\mu = \frac{(ln10)(0.4-0.2))}{372.01-336.21}$

$\mu = 0.01391\ min^{-1}$ $\mu = 0.01286\ min^{-1}$

And $\mu = \frac{(ln10)(0.4-0.2))}{5.86-5.31}$ $\mu = \frac{(ln10)(0.4-0.2))}{6.20-5.60}$

$\mu = 0.8344\ h^{-1}$ $\mu = 0.7718\ h^{-1}$

The selected range is always doubled cell division, from equation 4

Then $ln\frac{2N_0}{N_0} = \mu t$ when $N = 2N_0$ and $t = t_d$ as doubling time

$ln\,2 = \mu t_d$

Switch $t_d = \frac{ln2}{\mu}$ **… equation 10**

Put μ in equation 10;

ENM104

$t_d = \frac{ln2}{0.01391}$

$t_d =$ 49.83 min

SPS109

$t_d = \frac{ln2}{0.01286}$

$t_d =$ 53.87 min

Therefore, *P. pentosaceus* ENM104 had specific growth rate at 0.8344 h^{-1} and generation time at 49.83 min, while *L. plantarum* SPS109 also had specific growth rate at 0.7718 h^{-1} and generation time at 53.87 min.