การแยกและคัดเลือกแบคทีเรียแลคติคโพรไบโอติกที่มีคุณสมบัติลดคอเลสเตอรอลจากกากมันสำปะหลัง

นางสาวศิริวรรณ ณะวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2558

ISOLATION AND SELECTION OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM CASSAVA PULP FOR CHOLESTEROL LOWERING PROPERTY

Siriwan Nawong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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ISOLATION AND SELECTION OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM CASSAVA PULP FOR CHOLESTEROL LOWERING PROPERTY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ศิริวรรณ ณะวงษ์ : การแยกและคัดเลือกแบคทีเรียแลคติคโพรไบโอติกที่มีคุณสมบัติลดคอเลสเตอรอลจากกากมันสำปะหลัง (ISOLATION AND SELECTION OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM CASSAVA PULP FOR CHOLESTEROL LOWERING PROPERTY) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. รัชฎาพร อุ่นศิวิไลย์, 132 หน้า.

วัตถุประสงค์ของการศึกษานี้ เพื่อคัดแยก และระบุชนิดของแบคทีเรียโพรไบโอติกที่มีความทนต่อกรดและเกลือน้ำดี และมีคุณสมบัติในการลดปริมาณคอเลสเตอรอลจากกากมันสำปะหลังซึ่งเหลือทิ้งจากกระบวนการแปรรูปแป้งมันสำปะหลัง นอกจากนี้ได้ศึกษาผลของกระบวนการห่อหุ้มของเชื้อแบคทีเรียแลคติคโพรไบโอติกต่อการรอดชีวิตในระบบทางเดินอาหารจำลอง รวมทั้งอายุการเก็บรักษา ได้คัดแยกแบคทีเรียที่มีคุณสมบัติในการลดปริมาณคอเลสเตอรอลจากตัวอย่างกากมันสำปะหลังที่สุ่มเก็บในช่วงวันแรกจนถึงวันที่ 28 จากโรงงานอุตสาหกรมแป้งมันสำปะหลังในจังหวัดนครราชสีมา ประเทศไทย นำแบคทีเรียโพรไบโอติกที่คัดแยกได้ทั้งสิ้น 390 ไอโซเลท มาทำการทดสอบความสามารถในการเจริญในภาวะที่มีเกลือน้ำดีร้อยละ 0.15 และ 0.50 ทดสอบความสามารถเจริญในภาวะความเป็นกรด-ด่าง 2-9 และทดสอบความสามารถผลิตเอนไซม์ย่อยเกลือน้ำดีบนอาหารแข็งที่เติมเกลือน้ำดี (oxgall) ร้อยละ 0.3 พบว่าประมาณ 38 ไอโซเลท แสดงกิจกรรมของเอนไซม์ย่อยเกลือน้ำดี จากผลการทดสอบการลดปริมาณคอเลสเตอรอลของแบคทีเรียที่คัดเลือก 3 ไอโซเลท (3C2-10, 21C2-10 และ 21C2-12) พบว่าสามารถลดปริมาณคอเลสเตอรอลในอาหารเหลวได้ 18-24 ไมโครกรัมต่อมิลลิลิตร โดยเซลล์ที่มีชีวิตเท่านั้น และจากการทดสอบผลการใช้พรีไบโอติก พบว่า แบคทีเรียที่คัดเลือกสามารถใช้ ฟรุคโตโอลิโกแซคคาไรด์ (FOS), แลคตูโลส และ อินนูลินได้และมีความสามารถในการเกาะติดกับผนังลำไส้ได้ดี จากการวิเคราะห์ลำดับนิวคลีโอไทด์ 16S rRNA gene ของแบคทีเรียที่คัดเลือกได้ทั้ง 3 ไอโซเลท (3C2-10, 21C2-10 และ 21C2-12) พบว่าเป็น *Lactobacillus plantarum, L. acidophilus* และ *L. fermentum* ตามลำดับ

จากผลการศึกษาการเพิ่มความสามารถในการการรอดชีวิตในระบบทางเดินอาหารของมนุษย์จำลอง พบว่า สารสกัดจากกากมันสำปะหลัง แป้งข้าว และรำข้าว สามารถเพิ่มอัตราการรอดชีวิตในทางเดินอาหารจำลอง ของแบคทีเรียที่คัดเลือกได้ทั้ง 3 ไอโซเลท ประมาณ 7 log (CFU/มิลลิลิตร) ที่ภาวะความเป็นกรด-ด่าง 2 นาน 1 ชั่วโมง นอกจากนั้น นวัตกรรมการนำวัตถุดิบตามมาตรฐานอาหารเพื่อใช้ในการห่อหุ้มเซลล์ โดยการใช้ เจลาติน-มอลโตเดกตริน (G-MD) ร่วมกับเอนไซม์ทรานส์กลูตามิเนส (TGase) สามารถป้องกันแบคทีเรียโพรไบโอติกที่ถูกห่อหุ้มได้จากสภาวะระบบทางเดินอาหารจำลองเจลาติน-มอลโตเดกตริน (G-MD) ที่ทำจากเจลาติน เอ 300 และความเข้มข้นของเอนไซม์ทรานส์กลูตามิเนส (TGase) 10 ยูนิตต่อกรัม สามารถป้องกันการ

ทำลายของเซลล์แบคทีเรียที่ถูกห่อหุ้ม โดยเอนไซม์เปปซินในน้ำย่อยกระเพาะอาหาร (ที่สภาวะกรด-ด่าง 2 นาน 2 ชั่วโมง อุณหภูมิ 37 องศาเซลเซียส) และน้ำย่อยที่ลำไส้ (ที่สภาวะกรด-ด่าง 7.4 นาน 4 ชั่วโมง อุณหภูมิ 37 องศาเซลเซียส) จากการรอดชีวิตของแบคทีเรีย *Lactobacillus* ที่ถูกห่อหุ้ม (3C2-10, 21C2-10 และ 21C2-12) พบการลดลง 0.2-1 log (CFU/กรัม) เมื่อเปรียบเทียบการลดลงของเชื้อแบคทีเรียอิสระพบ 3-4 log (CFU/กรัม) การศึกษานี้แสดงให้เห็นถึงแนวโน้มของแบคทีเรียโพรไบโอติกที่คัดเลือกได้ทั้ง 3 ไอโซเลท มีคุณสมบัติในการลดระดับคอเลสเตอรอล

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SIRIWAN NAWONG : ISOLATION AND SELECTION OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM CASSAVA PULP FOR CHOLESTEROL LOWERING PROPERTY. THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 132 PP.

PROBIOTIC LACTIC ACID BACTERIA/ CASSAVA PULP/ CHOLESTEROL LOWERING PROPERTY

The objectives of this study were to isolate and identify potential probiotic bacterial strains from cassava pulp based on their acid and bile salt tolerances and cholesterol lowering activities. In addition, the influence of the microencapsulation process on the survival of potential probiotic lactic acid bacterial strains in simulated gastrointestinal conditions including storage time were studied. Cholesterol lowering probiotic bacteria were isolated from cassava pulp samples which were ramdomly collected from tapioca starch industries in Nakhon Ratchasima, Thailand from the first day to the 28thday. Three hundred and ninety isolates were tested for their tolerance on bile salt at concentrations of 0.15% and 0.50%, also from at pH 2 to 9 including bile salt hydrolase (BSH) activities were tested on plates containing 0.3% oxgall. Approximately 38 isolates showed BSH activity. Three selected strains (3C2-10, 21C2-10 and 21C2-12) could decrease cholesterol concentration in culture broth 18-24 µg/mL by only active cells. All selected strains (3C2-10, 21C2-10 and 21C2-12) showed the ability to metabolize prebiotics as Fructooligosaccharide (FOS), lactulose and inulin, and also showed the ability to strengthen cell adhesion. From 16S rDNA nucleotide sequence analysis, three strains (3C2-10, 21C2-10 and 21C2-12) were

identified as *Lactobacillus plantarum, L. acidophilus* and *L. fermentum,* respectively.

From the study related to the ability to survive in the simulated gastro-intestinal tract of selected strains, the results showed that the viability of three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) were improved by approximately 7 log (CFU/mL) in the presence of cassava pulp, rice starch and rice bran at pH 2 with 1h incubation time. In addition, a novel formulation of food-grade phase-separated gelatin-maltodextrin (G-MD) microspheres, where the gelatin was cross-linked with transglutaminase (TGase), would protect encapsulated probiotic lactic acid bacteria during exposure to the simulated upper gastro-intestinal tract conditions. The G-MD microsphere made with gelatin A300 bloom and a TGase concentration of 10 U/g prevented pepsin-induced degradation of the microspheres in simulated gastric juice (pH 2.0, 2 h , 37^{o}C) and intestinal juice (pH 7.4, 4 h , 37^{o}C). The survivor levels of the three encapsulated *Lactobacilllus* sp. (3C2-10, 21C2-10 and 21C2-12) were reduced by 0.2-1 log (CFU/g) as compard to 3-4 log (CFU/g) for the free non-encapsulated cells. The stability of the encapsulated process could provide protection for three strains of *Lactobacillus* sp. cells in refrigerated storage for 40 days. These studies demonstrated the potential of three selected isolates to be the probiotic lactic acid bacteria for cholesterol lowering property.

School of Food Technology Student's Signature_____

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Co-advisor's Signature_____

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Siriwan Nawong

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of varience
BLAST	=	Basic local alignment search tool
bp	=	Base pair
°C	=	Degree Celsius
CFU	=	Colony forming unit
DNA	=	Deoxyribonucleic acid
et al.	=	et alia (and others)
(m, μ) g	=	(milli, micro) Gram
h	=	Hour
(m, μ) l	=	(milli, micro) Liter
(m, μ) M	=	(milli, micro) Molarity
min	=	Minute
(m, μ) mol	=	(milli, micro) Mole
N	=	Normality
%	=	Percentage
PCR	=	Polymerase chain reaction
rpm	=	Round per minute
s	=	Second
SAS	=	Statistical analysis system
sp.	=	Species

LIST OF ABBREVIATIONS (Continued)

supsp.	=	Supspecies
%v/v	=	Percentage volume by volume
%w/v	=	Percentage weight by volume
W/O	=	Water in oil
O/W	=	Oil in water
U/g	=	Unit/g

CHAPTER I

INTRODUCTION

1.1 Introduction

1.1.1 Significance of the study

Cassava (*Manihot esculenta*) is one of the most important crops in terms of production and it was ranked as the sixth most important world food crop (Kaplinsky et al., 2011; FAO, 2015). In Thailand, cassava is a major product with around 20 - 25 million tons per year and about 12 percent of total world production (FAO, 2015). The domestic demand accounted for around 1.3-1.7 million tones but only 40 percent of the cassava produced in Thailand which processed into cassava starch (Kaplinsky et al., 2011). Cassava pulp is a fibrous by-product which is separated during the processing of cassava starch and usually used as animal feed. Cassava pulp contains about 50–60% starch on a dry weight basis, 1.5-5% protein, 0.1-4% fat and 10–15% fibers, including cellulose and other non-starch polysaccharides. The main of minerals in cassava pulp were Fe^{2+} of 155 ppm, Mn^{2+} of 40 ppm, $Mg^{2+}$of 1100 ppm, Cu^{2+} of 4 ppm, and Zn^{2+} of 21 ppm per kg-dry pulp (Julie et al., 2009).

The main problem in the drying process of cassava pulp is high starch and moisture content which are cause spoilage. Although cassava pulp is high in fermentable carbohydrates and moisture content but low in fiber and nitrogenous compounds. Due to cassava pulp is high organic nature, low cost and less competition with other industrial uses, thus it is a good substrate for microbial growth and production

of value added products (Rattanachomsri et al., 2009). It could thus be effect to the environment when it is not well managed and create environmental pollution problem.

The term ‘probiotic’ was first used in 1965 by Lily and Stillwell, described as substances stimulating the growth of other microorganisms. After the word probiotic was used in different meaning according to its mechanism and effect on human health (Mohamadshahi et al., 2014), the definition of probiotic was approved by FAO/WHO and International Scientific Association in 2001. The probiotic is defined as a live microorganism that when administered in adequate amounts exerts host health benefit (Ogueke et al., 2010; Morelli and Capurso, 2012). This definition is including mono or mixed culture of live microorganisms that are applied to animals and humans (Morelli and Capurso, 2012).

The most widely lactic acid bacteria strains used as probiotics in gastrointestinal tract usually belong to the *Lactobacillus* . The genus *Lactobacillus* has a long history of safety use including dairy foods and non-dairy foods application. Some physiological characteristics of *Lactobacillus* are of interest for their functions as probiotics, a pre-condition of which is survival in the gastrointestinal tract. This is based on their resistance to low pH and/or bile and temperature growth ranges. Moreover, their taxonomy, growth, nutritional requirements and metabolic activities clarify roles in the intestine or enhance their fermentative activity. The application of probiotic *Lactobacillus* strains has been extensively studied (Ejtahed et al., 2011; M Kumar et al., 2012; Kemgang et al., 2016). The probiotics could improve gut health and immunity, protect against harmful microorganisms, reduce infection severity, including show anti-hypertensive, anti-carcinogenic and obesity prevention (Ooi and Liong, 2010). *Lactobacillus* has been drew attention as microorganism with a potential for cholesterol- lowering in human. The serum cholesterol reduction is an important

health benefit such as a 1% reduction in serum cholesterol associated with an estimated of 2 to 3% reduction in the risk of coronary artery disease. The cholesterol reduction effect by *Lactobacillus* has been demonstrated in humans and animals (Haberer et al., 2003; Sirilun et al., 2010; R. Kumar et al., 2012). Cholesterol-lowering effects might be due to in part of de-conjugation of bile salts with enzyme bile salt hydrolase (BSH) produced by bacterial strain. As de-conjugated bile salts are more readily excreted in the feces than conjugated bile salts, *Lactobacillus* with BSH activity effectively reduce serum cholesterol by enhancing bile salts excretion, consequently increasing bile salts synthesis from serum cholesterol or decreasing the cholesterol solubility affecting its uptake from the gut (Massi et al., 2004; Liong and Shah, 2005; Khunajakr et al., 2008; Ramasamy et al., 2010).

The purposes of this research were to investigate the fundamentally screen, selection and identification of potential probiotic lactic acid bacteria isolated from cassava pulp and to find their cholesterol-lowering properties. Moreover, the physiology and morphology of selected strains were considered in relation to their viability after simulated gastric and intestinal juice model process which could lead for application as new probiotic for use in food products in the future.

1.2 Research objectives

The objectives of this research were as follows:

1) To isolate, select and identify potential probiotic bacterial strains from cassava pulp based on their acid and bile salt tolerance and cholesterol-lowering activities.

2) To increase the survival of potential probiotic lactic acid bacterial strains in

simulated gastrointestinal conditions.

3) To study the influence of encapsulation process on survival of potential probiotic lactic acid bacteria including storage time and condition.

1.3 Research hypotheses

A number of probiotic lactic acid bacterial species could be isolated from cassava pulp (cassava by-product). Some strains of these species would exhibit show acid and bile salt tolerance and cholesterol-lowering activities. The selected potential probiotic lactic acid bacteria would survive passage through similated upper gastro-intestinal tract conditions.

1.4 Scope of the study

Probiotic lactic acid bacteria were screened and isolated from cassava pulp based on their tolerante to acid and bile salt and cholesterol-lowering ability. The identification of probiotic lactic acid bacteria would be carried out (Massi et al., 2004; Sharma and Trivedi, 2015). Phenotypic and genomic analyse would be done using biochemical tests and/or the sequence analysis ampified by polymerase chain reaction (PCR) technique. The selected strains would prepared and studied for their survival in simulated gastrointestianl conditions. Influence of encapsulation process to survival and cholesterol-lowering activity of the selected strains would be studied, which also storage time of encapsulated strains would be investigeted.

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CHAPTER II

LITERATURE REVIEWS

2.1 Cassava utilization

Cassava, tapioca or manioc, is the common name of *Maniho esculenta Crantz* which was originated in South America and Southern and Western Mexico (Anyanwu et al., 2015). Cassava planted areas around the world was approximately 113.8 million crops with an average yield of 1.92 tons/crop (Kaplinsky et al., 2011). Nigeria, Brazil and Thailand are the major location of cassava productions in the world (Klanarong et al., 2012). However, Thailand had a higher yield of per area than that of Nigeria and Brazil. Thailand is currently one of the world's biggest exporters of cassava products with the major competitors Indonesia, Brazil and Vietnam (Kaplinsky et al., 2011). Cassava was planted in all regions of Thailand except in the South. More than 50% was planted in the Northeast, followed by the Central Plain (33%) and the North (15%). These planted areas included 48 provinces or around 7 million crops (FAO, 2015). There are around 150 cultivars of cassava. Each cultivar has various appearances, characteristics and quantities of hydrocyanic acid. A typical cassava root comprise of 60-70% moisture, 20-30% starch, 1% protein, 2% fiber, 1% oil and fat, 0.9-2.4% ash and 0.02% hydrocyanic acid (Román, 2016).

The cassava starch production is one of the most important agro-industries in Thailand. Thailand is the third largest producer of cassava starch in 2010, which are approximately yielded 22.5 million tons per year (Veiga et al., 2016). There are a 92

cassava starch factories which produce native (16,910 ton/day) and modified starch (4,350 ton/day) from cassava root (Virunanon et al., 2013). The utilization of cassava in Thailand can descried as Figure 2.1.

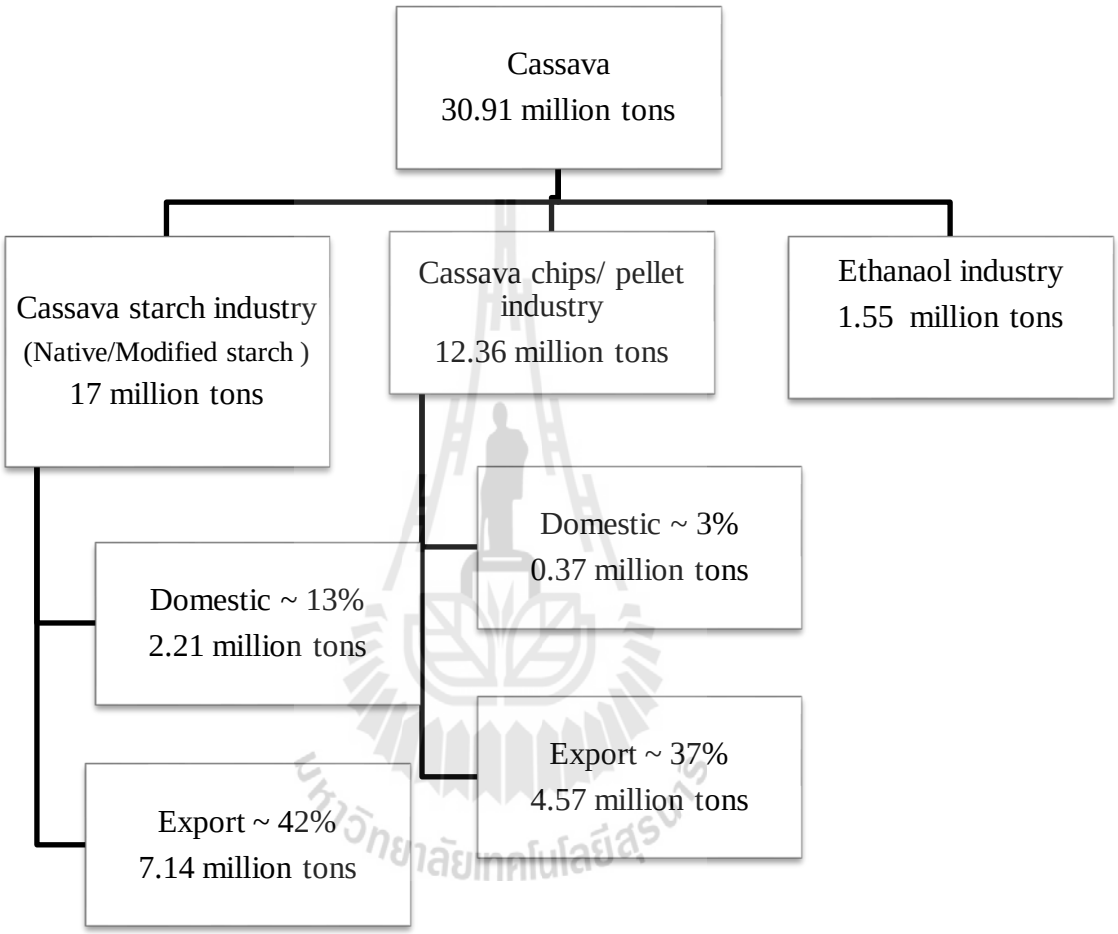

Figure 2.1 The cassava utilization in Thailand 2015.

From: Office of Agricultural Economics (OAE, 2015).

Tran et al., (2015) described the principle of cassava starch production oat small-scale factories (Figure 2.2), it begins with the extraction of starch granules from cassava root using water, which cassava roots are cleaning, peeling, chopping and grinding. Then, the starch slurry was filtered and sun-dried before the dried cake was

ground with grinding stone. Fiber and starch are separated by centrifugation to separate starch granules from protein and other impurities. The traditional process (small-scale) usually has a capacity around 1-5 tons per day and has insufficient starch product, not enough quality and capacity, and also high waste especially cassava solid wastes (Virunanon et al., 2013; Tran et al., 2015).

The solid wastes from cassava starch production are cassava stem, soil, sand, and pulp (Veiga et al., 2016). There is still lack of an interest in utilization these wastes for value adding. Nowadays, the wastes are mainly used as soil additive, animal feed and bio-alcohol production, etc (Virunanon et al., 2013; Anyanwu et al., 2015).

Cassava pulp is a residual pulp separated during the processing of cassava starch, and the main by-product from this process. Pulp is fine and white with moisture content up to 75% and the main composition is carbohydrate (55-56%)(Virunanon et al., 2013; Freire et al., 2015). Starch remain in the pulp approximately 50-60% of its dry weight, in which starch is trapped inside ligno-cellulose (Shigaki, 2016). The pulp also contains pectin, cellulose, and fiber approximately 10-15%, protein of 1.5-5%, fat of 0.1-4% (Kurdi and Hansawasdi, 2015; Shigaki, 2016). Other components are minerals: i.e., Fe^{2+} of 155 ppm, Mn^{2+} of 40 ppm, $Mg^{2+}$of 1100 ppm, Cu^{2+} of 4 ppm, and Zn^{2+} of 21 ppm per kg-dry pulp (Coulin et al., 2006; Kurdi and Hansawasdi, 2015; Shigaki, 2016).

Virunanon et al., (2013) has reported that around 25-30 million tons per year of fresh cassava roots are used for cassava starch production which generated cassava solid waste as cassava pulp around 3-5 million tones. High starch and moisture contents in cassava pulp cause problems in drying process and spoilage which affect environmental (Avancini et al., 2007). The pulp is high in fermentable carbohydrates

(Shigaki, 2016; Veiga et al., 2016) and moisture content but low in fiber and nitrogenous compounds (Freire et al., 2015). Due to its rich organic nature, this residue in cassava pulp could be a good substrate for microorganisms to grow and produce different products. Thus, it could affect the environment if it was not well managed leading to pollution problem.

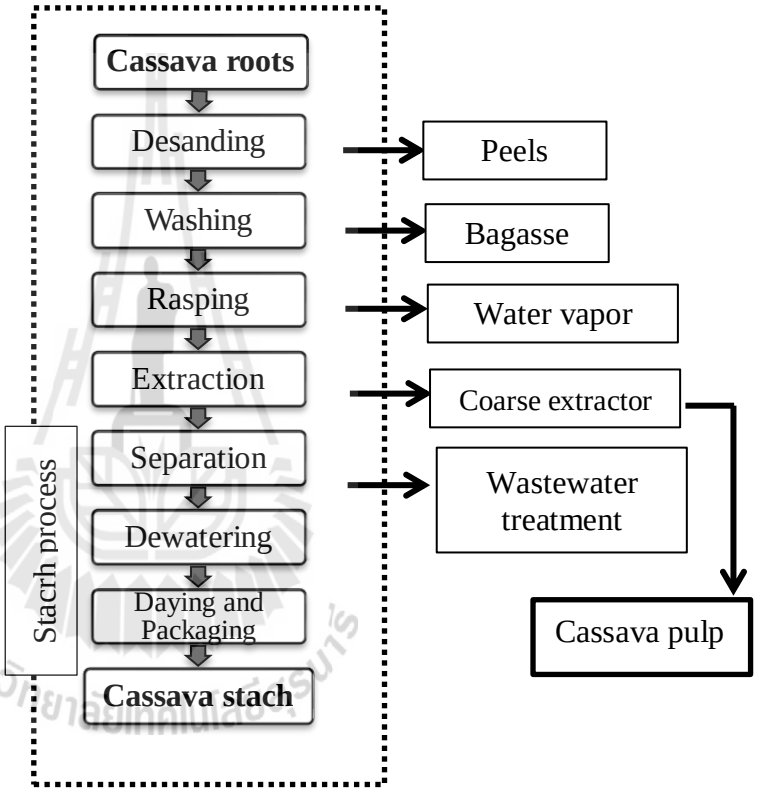

Figure 2.2 Diagram of the cassava starch manufacturing process in Thailand.

From: Tran et al., (2015).

The utilization of cassava pulp could be found in many ways, as used in animal feed industry, bio-gas production, and ethanol production. However, cassava pulp has a rich organic nature and low ash content, combined with the ease of its hydrolysis, low collection cost and lack of competition with other industrial uses, cassava pulp is

an ideal substrate for the value-added products.

2.2 Probiotic Lactic acid bacteria

Lactic acid bacteria (LAB) has a wide range of genera including an important number of species and generally recognized as being safe (GRAS) status for applications in foods (Bourdichon et al., 2012). LAB is a Gram-positive and generally catalase-negative bacteria (Zhang et al., 2011). They are non-spore forming and growing in microaerophilic to strictly anaerobic conditions (Ljungh and Wadstrom, 2006; Zhang et al., 2011). Endospore-forming lactic acid producing bacteria are aerobic to facultative aerobic and belong to the genera *Bacillus* and *Sporolactobacillus*. They are not LAB because of their biochemical and physiological characteristics (Björkroth and Koort, 2016). The most recognized genera of LAB are *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carnobacterium, Tetragenococcus*, and *Bifidobacterium* (Zhang et al., 2011; Ibrahim, 2016). Phylogenetically, Gram-positive bacteria is divided into two major branches. With the exception of the *Bifidobacterium*, all the above-mentioned genera of LAB belong to a Gram-positive phylum with a low G+C (guanine plus cytosine) content (50%) (Schleifer and Ludwig, 1995; Zhang et al., 2011; Björkroth and Koort, 2016; Mozzi, 2016). Nevertheless, LAB is focused on having similar physiological and biochemical properties and also association with the gastrointestinal tract (GIT). Species of these genera could be found in the GIT of man and animal as well as in fermented food. The strains used as probiotics usually belong to species of the genera *Lactobacillus, Enterococcus*, and *Bifidobacterium*. Essentially, chemoorganotrophic and fermented carbohydrate lead to the major end product from

lactic acid of LAB (Tamime, 2013). Some physiological characteristics of probiotic function are a pre-condition of cell survival in the gastrointestinal tract which is based on their resistance to low pH and/or bile (Šušković et al., 2000). Important physiological features are minor taxonomic that also considerate as resistance to different NaCl concentrations, carbohydrate fermentation patterns, growth on different nutrient medias, and their temperatures growth ranges including resistance to antibiotics. The taxonomy of Lactobacilli are based on these phenotypic properties for decades (Zhang et al., 2011; Björkroth and Koort, 2016).

Minor acid might be detected in some cultures such as acetic, succinic and formic (Coeuret et al., 2004; Tamime, 2013). The colony characteristic of some *Lactobacillus* strains is studied by Coeuret et al., (2004), the colony types of bacterial isolated from fermented milk show various colors and morphologies on MRS agar incubated anaerobically at 37 ^{o}C for 48–72 h. such as *L. acidophilus* show red with white color to translucent surrounding colony and circular. *L. casei* show red or white color to pale translucent and conical circular. While, *L. zeae* strain NCIMB 30096 and *L. rhamnosus* have star-shaped and convex circular.

Lactobacillus is the most widely used strains which has been approved as probiotics and applied in many food products over 40 countries for 50 years (Stiles and Holzapfel, 1997; Gill and Guarner, 2004; Miao et al., 2015). Some physiological characteristics of probiotics which interested are their functions as survivals in the gastrointestinal tract. This is based on their resistances to low pH and/or bile and temperature growth ranges. The genus *Lactobacillus* has a long history for safety use, including dairy foods, non-dairy foods, functional foods also mainstream food products (Emiliano et al., 2014; Nuraida, 2015). The taxonomy, growth, metabolic activities and nutritional requirements of *Lactobacillus* strains are clarified to their

roles in the gastrointestinal tract and enhance fermentative activity (Parisa et al., 2014; Pimentel et al., 2015; Björkroth and Koort, 2016).

In previous work, *Lactobacillus* strains were shown to inhibit pathogenic organisms, such as *Samonella*, *Shigella* and *Helicobacter* (Maragkoudakis et al., 2006; Suo et al., 2012; Emiliano et al., 2014). *Lactobacillus* strains could antagonize pathogenic bacteria by decreasing the luminal pH, translocation, inhibiting bacterial adherence or producing antimicrobial compounds such as bacteriocins (Miao et al., 2015; Björkroth and Koort, 2016). The major *Lactobacillus* species of the natural microflora in animal and human intestine are *L. acidophilus*, *L. plantarum* and *L. fermentum* ,etc. (Huang et al., 2015; Aoudia et al., 2016; Zhang et al., 2016). These species are presented in term of probiotics promoting a healthy equilibrium of microflora in the gastrointestinal system including preventing infection from pathogenic bacteria. Suo et al. (2012) found that *L. plantarum* strains ZJ316 showed antimicrobial activity against *Staphylococcus aureus*, *E. coli*, *Salmonella enterica*, *Bacillus subtilis*, *Listeria monocytogenes* and *Shigella dysenteriae*. Parisa et al., (2014) reported antimicrobial activities of *L. acidophilus* strain HM1 and *L. fermentum* strain HM2 against *Listeria monocytogenes, Staphylococcus aureus, Candida alicans, Shigella somei, Helicobacterer pylori, Enterobacter cloacae, Vibrio parahaemolyticus* and *Enterococcus faecium.* The ability to adhere to epithelial cells and mucosal surfaces of *Lactobacillus* strains has been referred to the action of Lactobacilli that could provide effective colonization on intestinal epithelial cells and can inhibit pathogen entry into the epithelial cells by bacteriocins production (Duary et al., 2011; Monteagudo-Mera et al., 2012).

The beneficial health concept of probiotic *Lactobacillus* strains have been proposed such as improving gut health and immunity, protection against harmful

microorganisms (Gill and Guarner, 2004; Galdeano et al., 2007; Homayouni, 2012). Moreover, probiotics *Lactobacillus* strains have been recorded to exert health promoting effects by several mechanisms. Some of these mechanisms are infection severity reduction, antihypertensive effects, anti-carcinogenic properties and cancer prevention especially colon and bladder, anti-oxidative effects, improving of arthritis, reduction of dermatitis and allergic symptoms (especially in infants and pregnant women), prevention of gastrointestinal disorders, dental carries, osteoporosis and obesity. However, *Lactobacillus* strains have also been studied for their cholesterol-lowering effects both *in vitro* and *in vivo* (Ouwehand, 2007; Jain et al., 2010; Yeo and Liong, 2010; Ejtahed et al., 2011). For several species of *Lactobacillus* especially, *L. acidophilus*, *L. plantalum* and *L. lactis* it has been published that the effects on cholesterol removal by varieties of mechanisms, however, these are inconclusive, yet.

The application of probiotic *Lactobacillus* strains have been studied. Lactic acid bacteria especially *Lactobacillus* sp. was used in fermented products in various materials including vegetables, fruits, cereals, soybeans, fish, meat and milk etc. (Nuraida, 2015). Some strains of *Lactobacillus* could be found in dairy and non-dairy products (Pimentel et al., 2015; Kemgang et al., 2016; Mohammad and Hashemi, 2016). Foods could be changed to be functional by probiotics addition. The productions of probiotics are important factor for the food substrate.

Coeuret et al. (2004) found that *L. casei* are available in some dairy products sale in Europe such as supplement feed, premix feed, fermented milk, Finnish emmental cheese and nutritional supplement that contains *L. casei* at levels of between 10^6-10^9 CFU/g. Some probiotic bacteria have been used in non-dairy products development such as fruits, vegetables, legumes and cereals. Five strains of *Lactobacillus* are found in vegetable materials such as Taiwanese pickled cabbage as

L. plantarum, *L.* reuteri, *L. paracasei*, *L. rhamnosus* and *L. acidophilus* (Chung-Yi et al., 2010). In addition, they were used in fruit juices with some prebiotics (as dietary fiber, cellulose etc. addition) which could induce ability of *Lactobacillus* to resist and an adapt stress response in acidic condition of fruit juices and survival longer during refrigerated storage (Gaanappriya et al., 2013; Marianne et al., 2015).

However, *Lactobacillus* strains used as probiotic, is a good choice and safe for food applications. The phenotypic and genotypic characteristics of probiotics are approved for safety aspects specifically *Lactobacillus* strains that show following characteristics:

1. Strains for human use are preferred to be of human origin.
2. They must be non-pathogenic.
3. They have no history of relationship with diseases such as infective endocarditis and/or gastrointestinal tract disorders.
4. They should not carry transmissible antibiotic resistance genes.

2.3 Prebiotics

Prebiotics are defined as non-digestible foods that are necessary for stimulating the growth of microflora in colon and improve host health (Gibson and Roberfroid, 1995). The major prebiotics used are carbohydrates and fibers such as resistant starch, wheat bran, inulin, polysaccharide and oligosaccharide (FOS; fructooligosaccharide, GOS; galactooligasaccharide, etc.) (Fang et al., 2007; Grimoud et al., 2010). Prebiotics have been suggested to give several beneficial to host such as promoting colonic microflora growth by stimulating proliferation, shortening oro-fecal transit time increasing, immunological response and also producing short chain fatty acid (Patel

and Goyal, 2010; Rastall, 2010). During fermentation process, colonic microflora could produce short chain fatty acids as well as CO_2, H_2 and CH_4 that affect anti-inflammatory, gene expression regulation, cell differentiation, and also serving as energy sources (Flint et al., 2007). However, the effect of prebiotic has been associated with bacterial growth conditions that colonic microflora and probiotic might compete for these prebiotics. Fang et al., (2007) explained that prebiotic as oligosaccharides could produce higher energy C-source than monosaccharides that lead to the bile resistance of *Lactobacillus* sp.

Currently, many food products in the market such as beverages, juice, chocolates, gums and infant foods etc. are fortified with prebiotics. The natural sources of prebiotics are preferred to reduce extra ingredients addition and also reducing cost of production. Moreover, the combination of prebiotics and probiotics has a synergistic interaction and called synbiotics shows advantage to consumers. Synbiotics are the combination of prebiotics and probiotics in a single product which attempts to obtain synergistic effects for improving human health (Saman et al., 2011). Synbiotics have been found to be more effective success in colonization or metabolic than probiotics or prebiotics alone (Grimoud et al., 2010). In previous studies, the effects of cereal grains were good substrates for intestinal microbiota and enhanced acid and bile tolerance (Gonzalez-Alvarado et al., 2008; Aumiller et al., 2015). Aumiller et al., (2015) reported that cereals were a new support of probiotic microorganisms because of their high content of essential vitamins, minerals and prebiotic fiber. Previously, cereal grains such as whole rice grains or fractions of the grain could be modified to improve their nutritional value or promote their functional properties (Saman et al., 2011).

In summary, prebiotics have a list of criteria for classification based on following properties: 1) resistance to host digestion and absorption in upper gastrointestinal tract. 2) fermentation by probiotics; and 3) selective stimulation of the growth and/or activity of probiotics that confers benefits host health and well-being (Gibson et al., 2004; Huebner et al., 2007; Rastall, 2010).

2.4 Acid and bile tolerance ability

Acid and bile tolerance ability is proposed to be a necessary characteristic of a probiotic strain that should survive and exert an action in the intestine tract. Generally, probiotic *Lactobacillus* strains are found in food system which begins with the mouth via the lower intestinal tract. *Lactobacillus* should be resistant to the enzymes such as lysozyme in oral cavity and pass through stomach and enter the upper intestinal tract containing bile. In this stage, *Lactobacillus* ought to resist the digestion processes, the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile range between 0.05-2% (Begley et al., 2006; Tambekar and Bhutada, 2010; Ruiz et al., 2013).

The acid and bile tolerance is the first criteria for probiotic bacteria selection. Bile acid are synthesized in the liver from cholesterol and stored at the gallbaladder and released into the duodenum in the conjugated form (approximately 500-700 ml/day) when diet intake by the host (Begley et al., 2005; Ruiz et al., 2013). Conjugated and deconjugated bile acids can affect bacterial growth exposed to their antimicrobial activity. Conjugated bile acid has more effect on Gram negative bacteria while Gram positive bacteria are more sensitive to deconjugated bile (Margolles et al., 2003). The morphological and physiological characteristics have been proposed for

screening bile tolerant strains. From previous studies, the morphology of *Lactobacillus* strains have been evaluated and showed morphology that was a parameter in the selection of bile salt-resistant probiotic *Lactobacillus* strains (Stiles and Holzapfel, 1997; Klein et al., 1998; Šušković et al., 2000). Šušković et al., (2000) found that the colonial and cellular morphology show a potential parameter to select bile salt tolerant *Lactobacillus* strains. The colony of *L. acidophilus* M92 was used in this study and exhibited heterogeneous colony types on MRS agar with and without bile. Smooth and rough colonies appeared in the culture, bile salts have an effect to rough colonies more than smooth colonies due to gaps in cell wall of round form that induced by the cytotoxicity of bile (Li, 2012). However, the colonies of microorganism is not a result of genetic variations, but rather phenotypic variability of the population caused by environmental conditions such as the presence or absence of oxygen, incubation temperature, pH, and growth medium components (Begley et al., 2005; Ruiz et al., 2013).

Normally, microorganism could not adapt to intestinal conditions due to bile is toxicity. The specific mechanisms resistance to the deleterious action by bile is found in enteric bacteria especially probiotic strains including *Lactobacillus* sp. The bile tolerance ability of Lactobacilli depends on species and type of strains (Mirlohi et al.,

2009; Ruiz et al., 2013). Blaiotta et al., (2013) reported that *L. casei* stain 1B has high survival rate (60.9%) in bile conditions than *L. casei* stain 5EG (1.1%). While *L. paracasei* subsp. *tolerans* strain 1DM showed strongly in bile conditions at 100% survival rate.

The selection of *Lactobacillus* strains based on acid stresses tolerance which is the most important characteristics of probiotics. It is crucial to understand the acid

resistance mechanisms. Liu et al., (2015) reported that the mechanism of microorganism adaptation in acid condition is based on maintaining on pH homeostasis during growth by F_0F_1-ATPase activity. Proton permeability is decreased to balance intracellular concentration of protons. The variation between strains and species of *Lactobacillus* strains to survive in acidic conditions was investigated. Liong and Shah (2005) established that the species of *Lactobacillus* reveals the ability of acid condition resistant. *L. acidophilus* showed higher cell viability (10^7 CFU/ml) than *L. casei* (10^4 CFU/ml) exposed to pH 2 for 2 h incubation. Mirlohi et al., (2009) reported that *L. acidophilus* Lac, *L. rhamnosus* Lk51, *L. rhamnosus* GG and *L. plantarum* A7 have been presented resisted to acid stress at pH 3 for 2 h (~10^8 CFU/ml) incubation and lost their viabilities in pH 2.5 for 2 h due to acidic environment. According to Tambekar and Bhutada (2010), five *Lactobacillus* strains were isolated from 40 goat milk samples illustrate *L. rhamnosus* G92, *L. plantarum* G95a, *L. plantarum* G96a, *L. rhamnosus* G99c and *L. rhamnosus* G119b. All isolates could grow in acidic to neutral range at pH 3-7 but were inhibited in alkaline at pH 9. Probiotic bacteria must pass through the stomach where pH could be low at levels of 1.5 to 3. This acid tolerance would help *Lactobacillus* to pass through to the small intestine and colon and thus contribute in intestinal microflora balancing (Xia et al., 2009; Ruiz et al., 2013). However, time of entrance to release from stomach is approximately 60 min, further digestive processes require longer residence time.

Therefore, it is essential that potential probiotic Lactobacilli should be able to grow in 0.15-0.3% bile at pH between1.5-3. Acid and bile tolerance ability is the way might useful for application in host environmental conditions and food systems.

2.5 Cholesterol-lowering property

2.5.1 Interactions of bile salts and probiotics

One of the important ability of probiotic is serum cholesterol reduction. Researchers reported that serum cholesterol level was related with risk of coronary heart disease. At 1% total serum cholesterol reduction could lower the risk of coronary heart disease by 2 to 3% (Guo et al., 2011; M Kumar et al., 2012). Cholesterol is a precursor of primary bile acid synthesized by liver. The primary bile acid form (chenodeoxycholic acid (CDCA) and Cholic acid) was changed to secondary bile acid form (lithocholic acid and deoxycholic acid) by enteric bacteria. Bile acid is conjugated with glycine or taurine by peptide-bond as Figure 2.3 (M Kumar et al., 2012; Li, 2012; Aydas. and Aslim, 2016). Conjugated and unconjugated bile acids are absorbed in the gut (passive diffusion) and terminal ileum (active transport). Conjugated bile acid is catalyzed by bile salt hydrolase enzyme (BSH) which hydrolyzes the peptide bond, resulting in conjugated bile acid form becoming converted to the unconjugated or deconjugated bile acid form which precipitate at low pH (Begley et al., 2006).

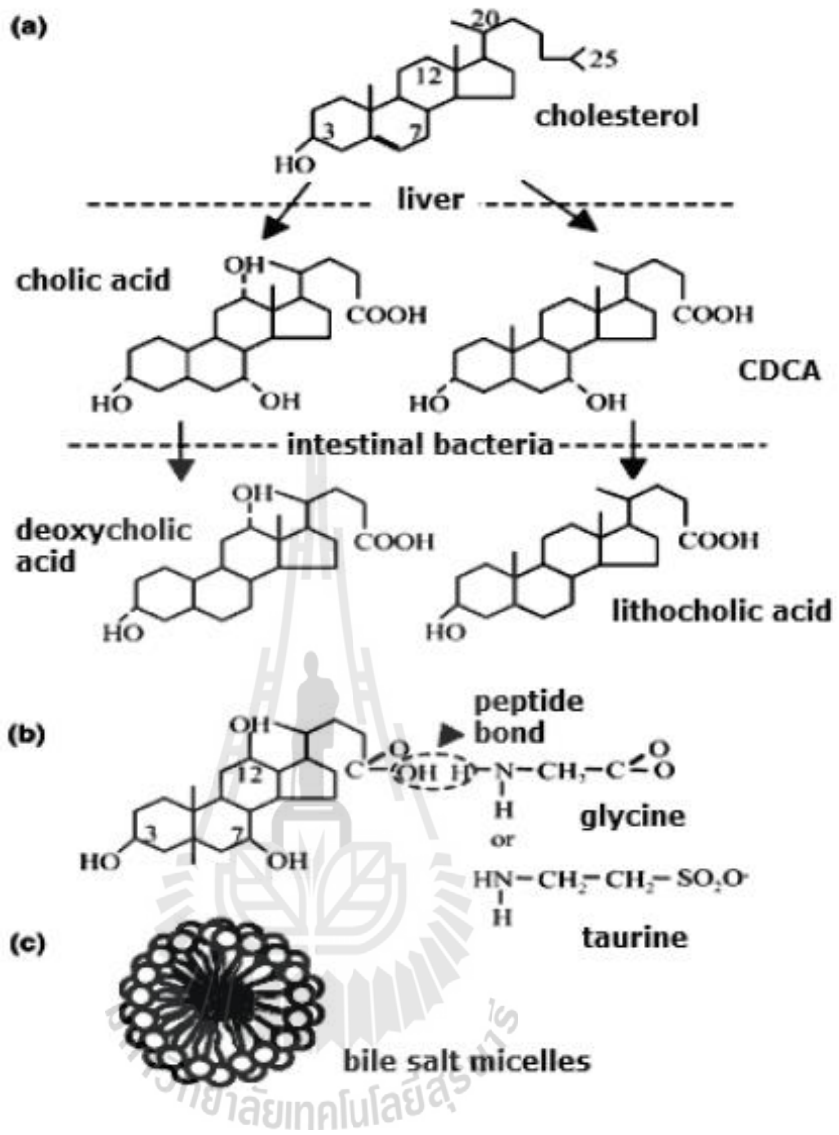

Figure 2.3 Chemical structures of bile acids in human digestive system

a) primary bile acid, b) secondary bile acid and c) bile salt micelles.

From: Li, (2012).

2.5.2 Cholesterol removing mechanisms of probiotics

The mechanism of cholesterol-lowering effect could not be explained definitely (Li, 2012; Aydas. and Aslim, 2016). Many researcher suggested that three hypotheses of cholesterol-lowering mechanisms of probiotics are mainly:1) deconjugation on bile

acids by bile salt hydrolysis (BSH) enzyme, 2) Co-precipitation of cholesterol with deconjugated bile acids and 3) Adsorption and incorporation of cholesterol to growing cell bacterial surface (Lim et al., 2004; Liong and Shah, 2005; Nguyen et al., 2007; Lye et al., 2010; Li, 2012; Aydas. and Aslim, 2016). Even though the current knowledge is inconclusive, yet, a large number of studies implied that the varieties of mechanisms might work together for lowering cholesterol level effect.

2.5.2.1 Effect of deconjugation of bile acids by BSH of probiotics

The cholesterol-lowering effects might be due to deconjugaton of bile acids by probiotic strains that produced BSH enzyme (Nguyen et al., 2007). BSH is an intracellular enzyme and exhibits in several colonic bacterial species such as *Lactobacillus* sp., *Bifidobacterium longum*, *Clostridium perfringens* and *Bacteroides fragilis* ssp. *fragilis* (Li, 2012; Zixing Dong et al., 2013). *Lactobacillus* spp. is a majority of bacterial bile salt hydrolase produced widely found in humans and animals intestinal tract and has been reported capability of cholesterol reduction using *in vitro* tests (Liong and Shah, 2005; Jones et al., 2013; Iranmanesh et al., 2014).

Probiotic strains with BSH activity might effectively reduce serum cholesterol by enhancing bile excretion, consequently, more bile could be synthesized from serum cholesterol, or by decreasing cholesterol solubility resulting in lowering absorption in gut system and large amounts of free bile acids form to be excreted in the feces (Kim and Lee, 2005; Sirilun et al., 2010; R. Kumar et al., 2012; Li, 2012).

Kim and Lee (2005) have been found BSH enzymes isolated from several *Latobacillus* strains but a few species of Lactobacilli showed absence of BSH enzyme activity as *Lactobacillus lactis*. Liong and Shah (2005) that found three strains of *Lactobacillus acidophilus* (ATCC33200, ATCC4357 and ATCC4962) excreted

BSH enzyme and had higher deconjugation ability than seven strains of *L. casei* (ASCC1520,

ASCC 279, ASCC 290, ASCC292, ASCC15820 and CSCC2607) correlated with that our results revealed ability to reduce cholesterol *in vitro* test. Kumar et al., (2012) reported that among 11 strains of *L. acidophilus*, 7 strains of *L. fermentum* and 33 strains of *L. plantarum* 100%, 50% and 42% tested positive for BSH activity, respectively. Noriega et al., (2006) suggested that the resistance to bile salts and deconjugation ability of their Lactobacilli were related with BSH activity and also conferred cross-resistance to enhance the survival of probiotic at low pH. Nguyen et al., (2007) isolated *L. plantarum* PH04 from infant feces which presented bile and acid tolerance and BSH activity. Moreover, *L. plantarum* PH04 excreted higher concentrations of BSH enzyme in stationary phase than exponential phase. The results indicated that *L. plantarum* PH04 affected hypercholesterolemic in mice when fed 10^7 CFU per mouse per day for 14 days which could reduce 7% serum cholesterol and 10% triglycerides in mouse.

Many researchers reported that BSH enzyme is responsible for the most important mechanisms that related to bile salt decondugation in the enterohepatic circulation (Parvez et al., 2005; Noriega et al., 2006; Lye et al., 2010; Jones et al., 2013). Generally, the biosynthesis is of major source of the cholesterol in the human body found in liver and absorbed in the intestines. Probiotics break down bile salts by synthesizing BSH enzyme which is able to hydrolyze conjugated bile salts, glycodeoxycholic acid and taurodeoxycholic acid, to the deconjugated of glycol bile acids and tauro bile acids form (Figure 2.4). Nevertheless, bile salts deconjugation by BSH enzyme from probiotic are possibility for hypercholesterolemia treatment in humans.

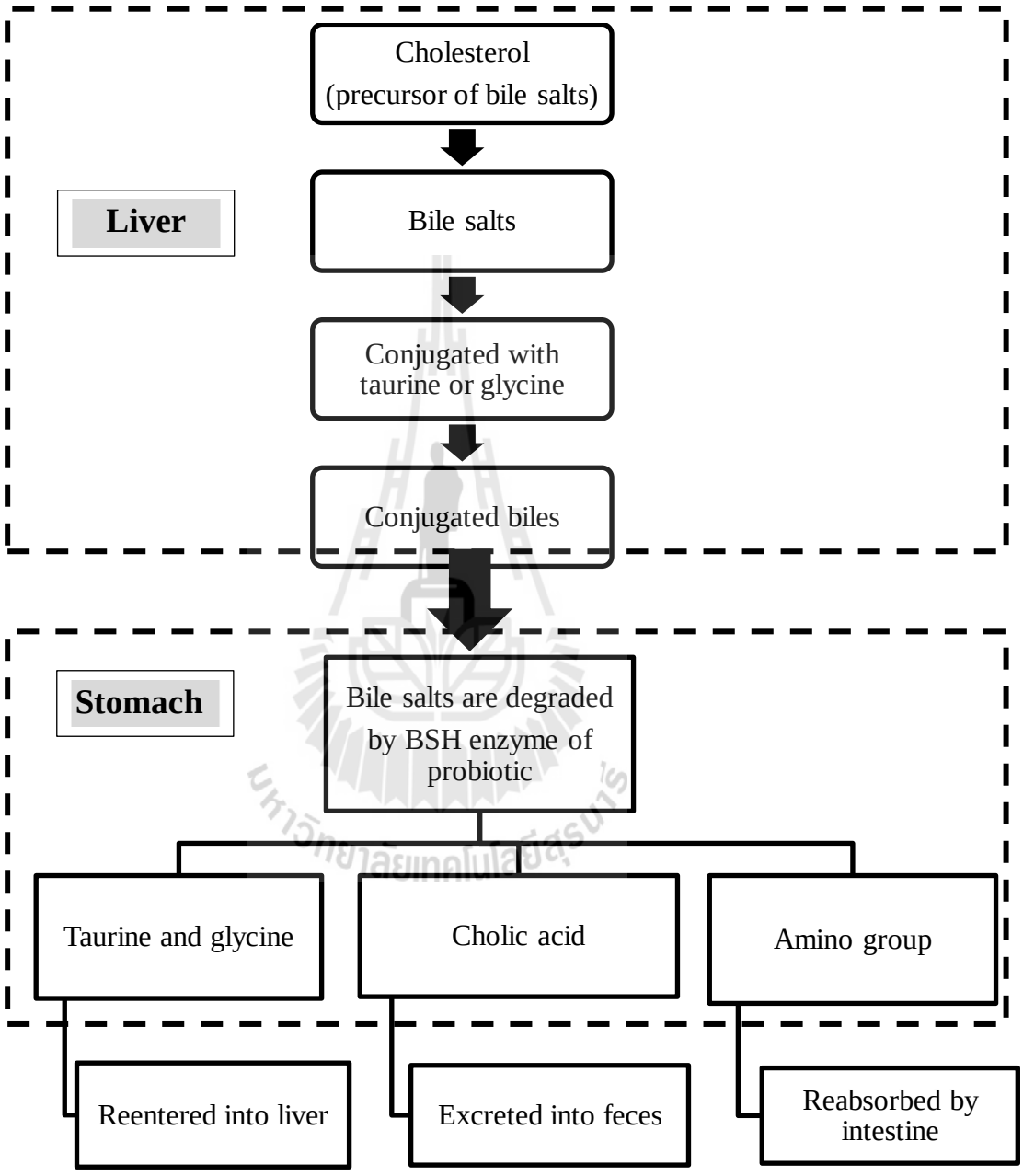

Figure 2.4 Cholesterol reduction mechanism of probiotic by bile salt hydrolase activity.

From: Kumar et al., (2012)

2.5.2.2 Effect of co-precipitation and assimilation of cholesterol with deconjugated bile

The mechanism of cholesterol assimilation was deconjugated bile salts co-precipitation with cholesterol particles at low pH (pH<5.5) which lead to cholesterol removal in intestinal systems (Aydas. and Aslim, 2016). Normally, the physiological pH of intestinal tract is slightly alkaline (about pH 8-9) which does not favor for co-precipitation reaction. The fermentation of probiotic in intestinal tract produces short-chain fatty acid which related to acidic conditions and established co-precipitation of cholesterol (Begley et al., 2006; Li, 2012). After deconjugated bile salts had co-precipitated with intestinal cholesterol in acidic conditions, cholesterol was removed by probiotic. Noriega et al., (2006) investigated the concentration conjugated bile salts that affected growth of *Bifiduacterium* strains and determined the cholesterol reduction by co-precipitation. Begley et al., (2006) had found that the assimilation ability depend on the probiotic growth and viability. Moreover, the assimilation shows stronger assimilate in active cells more than dead and resting cells (Liong and Shah, 2005; Sirilun et al., 2010; Li, 2012; Wesam et al., 2013). Sirilun et al., (2010) studied the cholesterol-lowering property of *L. plantarum* and reported that all four strains (TGCM15, TGCM26, TGCM33 and TGCM128) exhibited bile salts tolerance and BSH activity. Two strains of *L. plantarum* (TGCM 15 and TGCM 33) presented strong cholesterol reduction at pH between 4.37 and 4.22 respectively.

Co-precipitation and assimilation with deconjugated bile were related to the reduction of cholesterol levels. Lye et al., (2010) suggested that deconjugated bile salts (cholic acid) affected permeability to aqueous solutes in bacterial cell membrane

and induced membrane porosity which had more influence on the fluidity of outer and inner membrane layers, leading to assimilation of cholesterol.

2.5.2.3 Effect of binding and incorporation of cholesterol to bacterial cell surface

Cholesterol binding and incorporation of cell surface is another mechanism which probiotics could remove cholesterol from intestinal system (Kimoto-Nira et al., 2007; Li, 2012). Liong and Shan (2005) proposed that *L. casei* ASCC292 and *L. acidophilus* 4962 could remove cholesterol by incorporation of cholesterol to the cell surface during bacterial growth. Ramasamy et al., (2010) and Guo et al.,(2011) supported that *Lactobacillus* strains could significantly remove cholesterol from the growth medium. Kimoto-Nira et al., (2007) found that cholesterol is binding with cellular surface due to the chemical and structural properties of peptidoglycans in cell wall that composed of amino acids group and could bound to cholesterol via hydrogen bonds. Li (2012) reported that cholesterol was incorporated into the cell membrane of mycoplasma that could protect bacterial cells from lysis and increase the tensile strength of cell surface. Several studies concluded that cholesterol removal occurred from the binding of cholesterol and incorporation into the cell membrane during growth but not by cellular synthesis (Lye et al., 2010; Li, 2012; Bordoni et al., 2013; Aydas. and Aslim, 2016).

2.6 The response of *Lactobacillus* cells to human gastrointestinal conditions

2.6.1 Strain variation

One of the important roles of probiotic bacteria are survival at low pH of the stomach.

Normally, the pH on empty of human stomach is between 1-2 which increases to ranges 2.5-5 based on type of food consumption (Charalampopoulos et al., 2003; Xia et al., 2009; Sahadeva et al., 2011). Solid foods usually transit through the stomach around 2-4 hour, while liquid foods remain only 20 min (Annan et al., 2008). After *Lactobacillus* pass through the stomach and via the small intestine which it remains between 1 and 4 hour (Huang and Adams, 2004). The viability of probiotic bacterial cells after consumption indicated the resistance to gastrointestinal conditions including acid and bile secretions (Sahadeva et al., 2011). Various species of *Lactobacillus* have been reported to transit tolerance in gastrointestinal conditions but some species of some *Lactobacillus* strains are inhibited by the low pH of the stomach. According to the report of Michida et al., (2006) *Lactobacillus plantarum* NCIMB8826 demonstrated that cells decrease dramatically from 7.24 to 1.92 Log CFU/ml after incubation in gastric condition for 30 min without protective matrices (Michida et al., 2006). Cells number of *L. casei* subsp. *shirota*, *L. casei* subsp. *immunitas* and *L. acidophilus* subsp. *johnsonii* were also decreased under gastric condition by around 70-90% depending on factors such as type of species and physiological growth (Lo Curto et al., 2011). In the study of Blaiotta et al., (2013), five strains (1A, 4aM, 1S, 1DM and 4E) of *L. paracasei* subsp. *tolerans* were found to be strongly tolerant to low pH 2.5 which showed 54.8-100% survival (Blaiotta et al., 2013). *Lactobacillus* species are intrinsically acid tolerant although differences exist between specific strains and species, but it establishes high increased sensitivity at pH below 3 (Charalampopoulos et al., 2003).

The main challenges of *Lactobacillus* strains are viability in products and resistant to gastrointestinal tract conditions. The viability of probiotic bacteria was recommended in food products to be at least 6-7 Log CFU/g (Chotiko and Sathivel,

2016). Hence, it is necessary to provide high levels of live probiotic bacteria in food products and high survival level during the digestion processes to reach the site of action (large intestine) in high numbers which could provide health benefits.

2.6.2 Mechanism of bacterial survival to low pH

The ability to survive the gastric condition is an important criteria which selected a probiotic strains. Acid tolerance of bacterial are associated with the ability to control pH gradient between extracellular and cytoplasmic pH that inhibited cell and cell dies after the internal pH reaches a threshold value cellular functions (Charalampopoulos et al., 2003; Corcoran et al., 2005). The F_0F_1-ATPase is an important mechanism in the response and tolerance to low pH of Gram-positive lactic acid bacteria, which is used for protection against acidic condition and the action of control the H^+ concentration between the interior and exterior of the cell (Cotter and Hill, 2003; Xia et al., 2009). The F_0F_1-ATPase is a multiple-subunit enzyme consisting of a catalytic portion (F_1) incorporating the α, β, γ, δ, and ε subunits for ATP hydrolysis and an integral membrane portion (F_0) including the a, b, and c subunits, which together serve as a membranous channel for proton translocation (Sebald et al., 1982; Corcoran et al., 2005). The multi-subunit F_0F_1-ATPase is linked to the production of ATP though the transmembrane proton motive force and also generates ATP (Xia et al., 2009; Liu et al., 2015). The F_0F_1-ATPase plays an important role in organisms of living cells, when they lack a respiratory chain to generate a proton motive force through proton expulsion. At low extracellular pH, the F_0F_1-ATPase is induced and could increase the intracellular pH and regulation appears at the transcriptional level (Corcoran et al., 2005). The study of Xia et al., (2009) showed that *L. acidophilus* could survive in acidic conditions at low external pH <3.5 and showed ability to maintain cytoplasmic pH at values close to neutral.

2.7 Microencapsulation of probiotics to enhance viability

Microencapsulation techniques are relevant for food applications which are used to improve delivery of bioactive molecules and living bacterial cells into foods (Cook et al., 2012). Microencapsulation is a technique for entrap active agents in a carrier material and methods most widely used for probiotic bacteria are emulsion, extrusion, spray drying and freeze drying etc. (Nedovic et al., 2011). The necessary protection offered by microencapsulation for living cells could improve bacterial viability under specific conditions especially GI tract (Cook et al., 2012). The process of encapsulation, active agents is entrapped within a carrier material that used for as protective shell of encapsulates and should be food-grade, non-toxic or biodegradable (Nedovic et al., 2011). Generally, bacterial capsules will be micron sized and above with size ranges between 1-1000 µm (Singh et al., 2010). The carrier material is called the core material or bioactive which is surrounded by the wall material and sometimes may be double wall depending on the carrier material added (Birnbaum and Brannon-Peppas, 2004) . The core is packed in solid, liquid or gaseous materials in small capsules and can be released under specific conditions (Figure 2.5). The shape and sphere dimensions are based on the physico-chemical interactions between the wall and core and also the microencapsulation techniques (Cook et al., 2012).

Ideally, the microcapsule or microsphere should be designed to be semipermeable, spherical, strong and capable of retaining the bacterial cells until the controlled release under desired specific conditions. Also, the microspheres size should be small (less than <100 µm in diameter) so as not to affect the sensory attributes negatively when incorporated into foods.

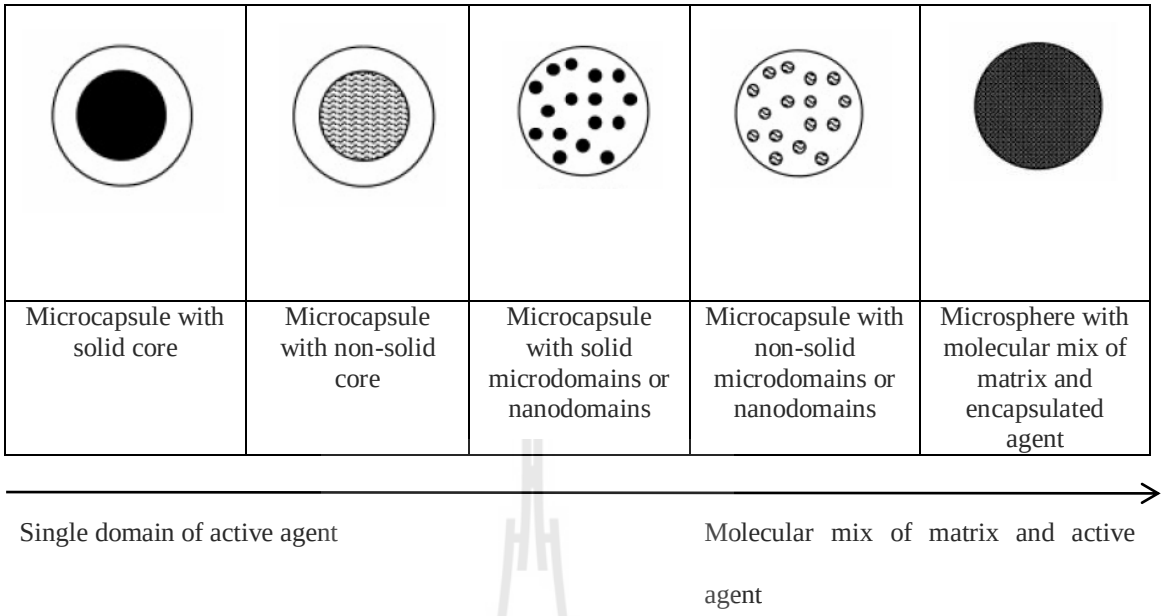

Figure 2.5 The different structures of microcapsules and microsphere.

From: Birnbaum and Brannon-Peppas, (2004).

Microencapsulation techniques can be applied for various food industries. The most important criteria of encapsulated product are functionality and stability requirements, should release at target site, provide living cells and cost constrains (Nedovic et al., 2011). Normally, spray drying is a flexible and continuous technique that is the most widely used in the food industry because it is more important an economic operation but there is disadvantages such as not being able to control particle size, high temperature during process and also complexity of equipment (Zuidam and Shimoni, 2010). In extrusion technique, it is popular technique due to low cost, ease and simplicity but it is difficult to scale up for large scale production (Krasaekoopt et al., 2003; Qiu-Yue Dong et al., 2013). Emulsion is another frequently technique that was found to be the best technique for industrial applications.

Advantages of these techniques are easy to scale up for large scale production and can control the size of capsule.

2.7.1 Encapsulation of probiotics by emulsion technique

Emulsion is a good technique that widely used for encapsulation of probiotic bacteria and can form capsules with diameters smaller than 1 mm (Mokarram et al., 2009). They are two combinations of emulsion as water in oil (W/O), oil in water (O/W) and also double emulsion as water/oil/water (W/O/W).

In this technique, the probiotic bacterial payload is essentially packed into microspheres composed of one or many different types of biologically compatible and non-toxic bio-polymers derived from natural sources such as seaweed (alginate, carrageenan), plants (starch and gum arabic), bacteria (gellan, xanthan), and animal (milk, gelatin), proteins (whey protein, soy protein, pea protein) (Chávarri et al., 2012; Qiu-Yue Dong et al., 2013; Wang et al., 2014). The size of microspheres is controlled by the speed of agitation and can vary around 25 µm to 2 mm. Moreover, this technique has been successfully applied for encapsulate probiotic lactic acid bacteria for batch and continuous process (Krasaekoopt et al., 2003; Mokarram et al., 2009; Qiu-Yue Dong et al., 2013).

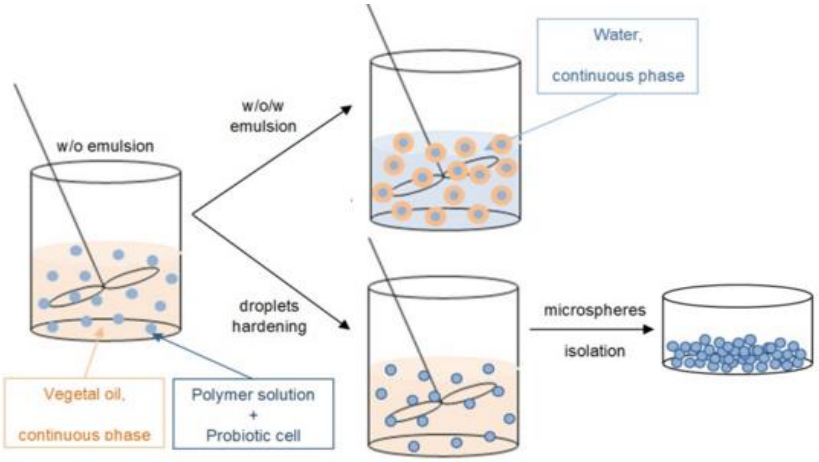

Figure 2.6 Encapsulation of emulsion technique by W/O and O/W.

From: Chávarr et al., (2012).

Several studies of emulsion technique have been proposed for enhance viability of probiotic bacteria. Borza et al., (2010) improved viability of *Bifidobacterium adolescentis* by modified W/O emulsion technique. *Bifidobacterium adolescentis* 15703T was encapsulated in multi-core microspheres which is combination between gelatine and maltodextrin, this modified technique increased survival of *B. adolescentis* 15703T cells in gastrointestinal conditions around 6-7 Log cycles. Zou et al. (2012) studied survival rates of microencapsulation into whey protein capsules by compared with spray drying and emulsion technique, the result showed that emulsion technique could significantly protect bacterial cells in acid condition better than spray drying technique. Moreover emulsion technique had high stability during storage at 4 ^{o}C and room temperature for 1 month. Recently, the emulsion method has been an effective method for protection in extreme processing condition at high temperature (72, 85 and 90 ^{o}C), high bile salt concentation (2%) and low pH in gastric condition (Sabikhi et al., 2008).

2.7.2 Gelatin-maltodextrin mixtures as potential microencapsulation materials

There are many materials which applied for supporting materials of the emulsion technique as alginate, starch, gum arabic, gellan, xanthan, milk, gelatin, whey protein etc. and including the mixture of materials (Qiu-Yue Dong et al., 2013; Wang et al., 2014). However, many materials need to show lack of cytotoxicity, safe biodegradations products and significantly to effect on the viability of bacteria.

Gelatin is biopolymer is produced by the partial hydrolysis of collagen and consists of protein (85-92%), mineral salts and water (Duconseille et al., 2015).

Gelatin is an approved and commonly used as ingredient in the food and pharmaceutical industry with potential application in microencapsulation formulations (Rokka and Rantamäki, 2010). However, microspheres formed by gelatin alone are physically weak and prone to be rapid enzymatic degradation by digestive enzymes. To overcome this problem, use of biocompatible cross-linkers such as genipin has been shown to stabilize gelatin based microspheres, thus enhancing the protection of entrapped bifidobacteria during exposure to simulated gastro-intestinal conditions (Annan et al., 2008). However, cross-linking with genipin resulted in the microspheres turning dark blue thus limiting their potential use in food products.

Maltodextrin is a neutral polysaccharide polymer that derived from starch hydrolysis and has a good source for stimulating bacterial growth (Borza et al., 2010). Corcoran et al., (2005) found that metabolize sugars enhanced survival of probiotic bacteria in acidic conditions by maintaining ATP and permitting optimal proton (H^+) extrusion by F_0F_1-ATPase. Presence of maltodextrin increases the yield of live and functional probiotic lactic acid bacteria cells after exposure to thermal stress during spray drying (Reddy et al., 2009). Enhanced protection during simulated upper GI tract transits was also observed for *Bifidobacterium adolescentis* encapsulated in genipin cross-linked gelatin-maltodextrin (G-MD) microspheres in comparison to survival in gelatin only microspheres (Borza et al., 2010). The promise of the G-MD matrix for microencapsulation led us to search for alternative non-toxic and colourless gelatin cross-linkers.

Transglutaminase (TGase, EC. 2.3.2.13), which catalyses an acyl-transfer reaction between the carboxyamide group of peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the amino group of lysine residues to form an ε-(γ-glutamyl) lysine isopeptide bond (Yung et al., 2007; Zou et

al., 2012), fulfills these requirements. The GRAS status of TGase has meant that the enzyme is widely used in different food products to improve functional properties such as hydration, stability of gels and emulsions or foaming properties of soybean proteins (DeJong and Koppelman, 2002; Cho et al., 2003; Borza et al., 2010; Pavunc et al., 2011; Zou et al., 2011). In a previous study, a microencapsulation method was developed using TGase-induced gelation of caseinate for probiotic cells protection and shown to enhance viability probiotic cells during processing, storage and including gastrointestinal transit (Heidebach et al., 2009). Zou et al., (2012) reported that microencapsulation process developed from whey protein microcapsules that produced by TGase-induced gelation method had a significantly protective effect of *Bifidobacterium bifidum* F-35 in simulated gastric juice (SGJ) at low pH conditions and remained stable during storage at 4^{o}C or at room temperature during 1 month.

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CHAPTER III

ISOLATION AND SELECTION OF CHOLESTEROL-LOWERING PROBIOTIC LACTIC ACID BACTERIA FROM CASSAVA PULP

3.1 Abstract

Cholesterol-lowering probiotic bacteria screening from cassava pulp was studied. The cassava pulp samples were randomly collected from tapioca starch industries in Nakhon Ratchasima, Thailand during day zero to day 28th. The isolation, selection and identification of potential probiotic bacterial strains from cassava pulp were conducted based on their acid and bile salt tolerance including *in vitro* cholesterol-lowering properties. The isolation was done under anaerobic conditions at 37°C with De Man Rogosa Sharpe (MRS) agar. The isolates were tested for their tolerance on bile salt at concentration of 0.15% and 0.50%, also at pH 2 to 9. Bile salt hydrolase (BSH) activities were tested on plates containing 0.3% oxgall. Thirty-eight isolates from 390 isolates showed precipitation zones on plate with 0.3% oxgall which indicated BSH activity. Three selected strains (3C2-10, 21C2-10 and 21C2-12) could decrease cholesterol concentration in culture broth by 18-24 µg/mL by only active cells. Identification of probiotic lactic acid bacterial isolates was performed by using API 50 CHL test kits and were confirmed using partial 16S rDNA sequence analysis. Three strains showed high BSH activities and cholesterol-lowering properties; 3C2-10, 21C2-10 and 21C2-12, were identified as *Lactobacillus plantarum, Lactobaillus*

acidophilus and *Lactobacillus fermentum,* respectively. These strains also tolerated 0.15% and 0.50% (w/v) bile salt and resisted to pH level 3-9 with survival during 2 h growth on MRS medium. In addition, strains 3C2-10 exhibited strong antimicrobial activities against *Enterobacter aerogenes* bcc6719, *Bacillus subtilis* TISTR008, *Escherichia coli* TISTR3436, *Bacillus cereus* TISTR687, *Pseudomonus aeruginosa* TISTR781 and *Staphylococcus aureus* TISTR1466. All selected strains (3C2-10, 21C2-10 and 21C2-12) showed strength of cell adhesion and the ability to metabolize prebiotic as fructooligosaccharide, lactulose and inulin. All strains exhibited BSH activities and *in vitro* cholesterol-lowering property. In conclusion, the 3 isolated strains (*L. plantarum*, *L. acidophilus* and *L. fermentum*) all were promising and exhibited the potential to lower cholesterol *in vitro*.

Keywords: Cholesterol-lowering bacteria, Cassava pulp, *Lactobacillus* sp., Probiotic, Bile salt hydrolase (BSH).

3.2 Introduction

The domestic demand for cassava starch is as high as around 1.3-1.7 million tons per year. However, only 40 percent of the cassava produced in Thailand is used for cassava starch production (Kaplinsky et al., 2011). During the processing of cassava starch, cassava pulp is a fibrous by-product of the cassava processing industry, and generally used as low-value animal feed. Fifty percentage (w/w) of cassava pulp mainly contains cellulose and other non-starch polysaccharide and other components as 1.5-5% protein, 0.1-4% fat and 10–15% fibers. The pulp also contains minerals such as Fe^{2+} of 155 ppm, Mn^{2+} of 40 ppm, $Mg^{2+}$of 1100 ppm, Cu^{2+} of 4 ppm, and Zn^{2+} of 21

ppm per kg-dry pulp (Coulin et al., 2006). Because of cassava pulp is rich in organic substance with low ash content that is low cost, cassava pulp is an ideal substrate for the microbial production of value-added products (Coulin et al., 2006; Kaplinsky et al., 2011). Finally, cassava pulp could cause the environment pollution if it was not suitable managed.

The term 'probiotic' was firstly used in 1965 by Lily and Stillwell, described as substances stimulating the growth of microorganisms in human gastrointestinal tract. After, the word probiotic was used in different meaning according to its mechanism and effect on human health (Mohamadshahi et al., 2014), the definition of probiotic was approved by FAO/WHO and International Scientific Association in 2001. The probiotic is defined as a live microorganism that when administered in adequate amounts exert host health benefit (Ogueke et al., 2010; Morelli and Capurso, 2012). This definition is including mono or mixed culture of live microorganisms that have been used in animal and human (Morelli and Capurso, 2012).The most widely lactic acid bacteria strains used as probiotics in gastrointestinal tract usually belong to the genera *Lactobacillus* species. The genus *Lactobacillus* has a long history of safety use including dairy foods and non-dairy foods application. Some physiological characteristics of *Lactobacillus* are of interest for their functions as probiotics, a pre-condition of which is survival in the gastrointestinal tract. This is based on their resistance to low pH and/or bile and their temperature growth ranges. Moreover, their taxonomy, growth, nutritional requirements and metabolic activities clarify their roles in the intestine or enhance their fermentative activity. The application of probiotic *Lactobacillus* strains has been studied. The probiotics could improve gut health and immunity, protect against harmful microorganisms, reduce infection severity, including show anti-hypertensive, anti-carcinogenic and obesity prevention properties (Ooi and

Liong, 2010). *Lactobacillus* has drawn attention as microorganism with a potential to be cholesterol-lowering in human. The serum cholesterol reduction is an important health benefit as a 1% reduction in serum cholesterol is associated with an estimated of 2 to 3% in the risk of coronary artery disease reduction. The cholesterol reduction by *Lactobacillus* has been demonstrated in human and animals (Haberer et al., 2003; Sirilun et al., 2010; Kumar et al., 2012). Cholesterol-lowering effects might be from de-conjugation of bile salts with enzyme bile salt hydrolase (BSH) that is produced by bacterial strain. As de-conjugated bile salts, it is more readily excreted in the feces than conjugated bile salts, *Lactobacillus* with BSH activity effectively reduce serum cholesterol by bile salts excretion enhancement, consequently increasing bile salts synthesis from serum cholesterol or decreasing the cholesterol solubility affecting its uptake from the gut (Massi et al., 2004; Liong and Shah, 2005; Khunajakr et al., 2008; Ramasamy et al., 2010). The research objectives were to investigate the isolation, selection and identification of potential probiotic lactic acid bacteria that show lower cholesterol *in vitro*. The cassava pulp was used as the source of the bacterial isolates.

3.3 Materials and methods

3.3.1 Collection of cassava pulp (cassava by-product) samples

Cassava pulp samples were randomly collected from tapioca starch industries (Sa-nguan Wong Industry Co., Ltd.) in Nakhon Ratchasima, Thailand. Cassava pulp samples were ensilaged at various times (0, 1, 3, 5, 7, 14, 21 and 28 days) under anaerobic conditions at 37^{o}C (Jintanawit et al., 2006).

3.3.2 Isolation and screening of probiotic lactic acid bacteria from cassava pulp samples

Probiotic lactic acid bacteria were isolated and screened from cassava samples (Homayouni et al., 2012). De Man Rogosa Sharpe agar (MRS) was used for probiotic lactic acid bacteria isolation (De Man et al., 1960). All plates were incubated under anaerobic condition at 37°C for 48 h. Colonies were arbitrarily collected based on the differences in colony morphology. Purified bacteria were maintained on the same medium at 4°C. The purified isolates were activated and subcultured on the MRS medium and incubated at 37°C under anaerobic condition for 48 h.

3.3.2.1 Determination of bile salt hydrolase (BSH) activity

The BSH activity method was analyzed (du Toit et al., 1998; Lim et al., 2004). Selected strains were tested for BSH activity using MRS agar containing 0.3% oxgall and incubated at temperature 37°C under anaerobic condition for 48-72 hours. The colonies which induced precipitation zone were scored positive for BSH activity.

3.3.2.2 Determination of acid and bile salt tolerance

The isolated strains were inoculated into MRS broth at various pH (2, 3, 4, 5, 8 and 9) and incubated at 37°C under anaerobic condition for 2 h. The strains that grew at various pH were counted by spread plate technique on MRS agar (37°C,48 h). Bile salt tolerance was tested in MRS agar with bile salt at the concentration of 0.15 and 0.50% and incubated at 37°C under anaerobic condition for 48 h. The strains that grew in the bile salt were scored positive for bile salt tolerance.

3.3.2.3 Determination of antagonistic activity

The isolates were screened for antimicrobial-producing activity (Cizeikiene et al., 2013). The isolates were cultured in MRS broth supplemented with 2.5% yeast extract and spotted onto MRS agar. Plates were incubated for 24-48 h until growth was evident, after that overlaid with 5 mL trypticase soft agar (0.7% agar) containing 0.1 mL of an overnight culture of *Enterobacter aerogenes* bcc6719,

Bacillus subtilis TISTR008, *Escherichia coli* TISTR3436, *Bacillus cereus* TISTR687, *Pseudomonus aeruginosa* TISTR781 or *Staphylococcus aureus* TISTR1466. Plates were incubated at 37°C for 18-24 h after that the clear zones around spots were measured and compared with positive control. Chloramphenicol was used as a positive control.

3.3.2.4 Determination of cell adhesion of potential probiotic lactic acid bacteria

The adherence of three selected *Lactobacillus* strain to the Caco-2 cell lines were analyzed (Duary et al., 2011; Monteagudo-Mera et al., 2012).

The human colonic carcinoma cell line Caco-2 (ATCC®,HTB-37™, American Type Culture Collection, Manassas, VA, USA) was cultured in the complete medium. The complete medium contained Dulbecco's modified Eagle's minimal essential medium (DMEM; GIBCO® cat.no. 11965-092, Thermo Fisher Scientific Inc.), 10.0% (v/v) heat-inactivated (30 min, 56 °C), fetal bovine serum (FBS; GIBCO® cat.no. 10270-098, Thermo Fisher Scientific Inc.), 1% (v/v) penicillin-streptomycin (Pen-Strep; GIBCO® cat.no. 15140-122, Thermo Fisher Scientific Inc.), 1% (v/v) non-essential amino acids (MEM NEAA; GIBCO® cat.no. 11140-050, Thermo Fisher Scientific Inc.) and 1% (v/v) L-glutamine (GIBCO® cat.no. 25030-081, Thermo Fisher Scientific Inc.). The cell cultures were incubated at 37°C in sterile plastic tissue culture flasks T-75 (75 cm^2 cell culture flask) with 5% CO_2 atmosphere and replaced by fresh complete medium every 2 days. Caco-2 cells were used at 80% confluency after 20 days of culture.

The adhesion assay of *Lactobacillus* strains to the Caco-2 cell line was tested (Monteagudo-Mera et al., 2012). Bacterial cells cultures were resuspended in DMEM and transferred to sterile plastic tissue culture flasks T-25 (25 cm^2 cell culture

flask) and incubated at 37°C for 2 h in 5% CO_2 atmosphere. After incubation, sterile phosphate-buffered saline (pH 7.4, PBS; GIBCO®, cat.no. 70011, Thermo Fisher Scientific Inc.) was used for washing the unattached bacteria. The adhered bacteria were measured by plate count on MRS Agar.

Adhesion values (%) were calculated as follows:

$$\%Adhesion = \frac{V1 \times 100}{V0}$$

Where V0 is the initial viable count of bacteria tested, and V1 is the viable bacteria count obtained from the Caco-2 cells.

3.3.2.5 Determination of cell surface hydrophobicity

Cell surface hydrophobicity was evaluated by microbial adhesion to hydrocarbons (MATH) method (Kotzamanidis et al., 2010; Duary et al., 2011). Washed cell suspensions of three *Lactobacillus* strains were resuspended in 5 mL of PUM buffer (K_2HPO_4: 22.2 g/l; KH_2PO_4: 7.26 g/l; urea: 1.8 g/l; $MgSO_4$: 0.2g/l; pH 7.1 ± 0.2) and measured at absorbance 600 nm (A0). One milliliter of hexadecane or iso-octane was added to 5 mL of cell suspension and vortexed for 2 min. After 1 h incubation at 37ºC to allow the phase separation. The aqueous phase was carefully removed and absorbance was measured at 600 nm (A1). The percentage of cell surface hydrophobicity (%H) was calculated using the formula:

$$\%H = \left(\frac{1 - \mathrm{A1}}{\mathrm{A0}}\right) x\ 100$$

Where A0 is the initial absorbance at 600 nm of cell suspension, and A1 is the aqueous phase absorbance at 600 nm. Cells were scored into three groups.

H% > 70 **(**High hydrophobicity)

H% = 50-70 **(**Moderate hydrophobicity**)**

H% < 50 **(**Low hydrophobicity**)**

3.3.2.6 Determination of prebiotic activity

Prebiotic activity was tested followed method described by Huebner *et al*., (2008). Three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) were cultured in MRS-broth with 2% prebiotics. Cassava pulp, Fructooligosaccharide (FOS; Sigma Aldrich®, Saint Louis, MO, USA), Lactulose (LAC; ACROS Organics™, Thermo Fisher Scientific Inc, New Jersey, USA), Inulin (Sigma Aldrich®, Saint Louis, MO, USA) and Cellulose (HiMedia® Laboratories Pvt Ltd., Mumbai, India) were tested and incubated 37°C under anaerobic condition for 20 h.

The prebiotic activity score was determined following equation:

$$\text{Prebiotic activity score} = \left[\frac{(\text{probiotic log CFU on the prebiotic at 24 h} - \text{probiotic log CFU on the prebiotic at 0 h})}{\textit{probiotic} \text{ log CFU on glucose at 24 h} - \text{probiotic log CFU on glucose at 0 h})}\right] - \left[\frac{(\text{enteric log CFU on the prebiotic at 24 h} - \text{enteric log CFU on the prebiotic at 0 h})}{\text{enteric log CFU on glucose at 24 h} - \text{enteric log CFU on glucose at 0 h})}\right]$$

3.3.3 Determination of *in vitro* cholesterol-lowering activity

The selected strains were investigated for cholesterol-lowering activity was modified by Mojgani *el al*., (2015) and Sirilun *et al*., (2010).

Growth culture of strains (1%) was inoculated into freshly prepared MRS supplemented with 0.3% oxgall (w/v) (Sigma, USA) as a source of bile salt. Standard cholesterol in water-soluble form (polyoxyethanyl cholesteryl sebacate; Sigma, USA) was sterilized, filtered and added to MRS broth at a final concentration of 100 mg/L. After 24 h of growth, the final pH of active cell culture was measured. Then, the cells were centrifuged. Spent broth was then sterilized by using 0.2 µm membrane filter (Corning, NY 14831, Germany) as cell-free broth of active cells. The total amount of cholesterol in both the active cell-free broth and cell pellet were determined. An un-

inoculated MRS broth at the same condition was a negative control.

Active cells were prepared on MRS broth containing with 0.3% oxgall as a source of bile salt. Standard cholesterol in water-soluble form (polyoxyethanyl cholestery sebacate; Sigma Aldrich®, Saint Louis, MO, USA) were sterilized, filtered and added to MRS broth at a final concentration of 100 µg/mL. After incubation for 20 h at 37°C anaerobic condition, the final pH of active cell culture was measured. Cells were centrifuged (10,000 rpm for 10 min). Supernatant (spent broth) was sterilized by using 0.45 mm membrane filter as cell-free broth of active cells. The total amount of cholesterol in the active cell-free broth and active cell-pellet were determined. An un-inoculated MRS broth at the same condition was used as a negative control.

Inactive cells dead cells were prepared from MRS broth containing 0.3% oxgall and incubated for 20 h at 37°C anaerobic condition. The inoculation was the used cell harvested by centrifuging at 10,000 rpm for 10 min. Cell pellets were washed twice with sterile distilled water, subsequently suspended in sterile distilled water and autoclaved at 121°C for 15 min. After being autoclaved, the cell pellets were suspended in MRS broth containing 0.3% oxgall and water-soluble cholesterol at a final concentration of 100 µg/mL.

The active cells, cell pellets and dead cells were incubated at 37° C for 20 h. Spent broths were assayed for cholesterol-lowering activity following *o*-phthalaldehyde method (Gilliland et al., 1985). The active cells were also confirmed measurement following cholesterol oxidase/peroxidase method using Automated BioSystems (BioSystems S.A. Costa Brava30, Barcelona, Spain) by Suranaree University of Technology Hospital.

$$\text{Percentage of cholesterol} - \text{lowering } (\%) = \left[1 - \frac{[(\text{residual cholesterol in cell free broth})]}{(\text{cholesterol in control broth})}\right] x100$$

3.3.4 Identification of potential probiotic lactic acid bacteria

The isolates were identified based on morphological and biochemical characteristics (Massi et al., 2004; Sharma and Trivedi, 2015). Identification of probiotic lactic acid bacterial isolates was performed using API 50 CHL test kits, (BIO-Merieux, Marcy-I 'Etoile, France) the result was analyzed based on carbohydrate fermentation pattern using api-web program. The partial 16S rRNA gene was amplified use the polymerase chain reaction (PCR) technique using Primers (16UNI-L f/ 16UNI-R r) at product size 1300 bp. The genomic DNA extraction was done following the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Direct sequence of 16S rRNA gene products was performed. Then, DNA sequencing was performed (McGill University and Gemone Quebec Innovation Centre Canada). The bacterial sequences were compared to local alignment search of the GenBank database using the BLAST program of the National Center for Biotechnological Information (NCBI).

3.3.5 Scanning electron microscopy examination

The attachment of cholesterol onto selected strains were observed using scanning electron microscopy (SEM). The cell pellet was prepared (Huey-Shi et al., 2010). The morphology of gold coated samples were examined at 10.0 kv (Auriga FIB-SEM, ZEISS, Germany).

3.3.6 Statistical analysis

Results were statistically analyzed where appropriate by Analysis of Variance (ANOVA) using the SPSS 16.0 software package for Windows.

3.4 Results and discussion

3.4.1 Isolation of probiotic lactic acid bacteria from cassava pulp samples

Lactic acid bacteria isolated from cassava pulp samples during zero to 28th day of fermentation amounted to approximately 6-7 log CFU/g at pH around 4 (Table 3.1). The number of microorganisms was about 7 log CFU/g at zero to 7th day, and decreased by around 1 log CFU/g after fermentation for 7 days. These results were similar to those studied by Coulina *et al*., (2006) who found that lactic acid bacteria was dominant in cassava pulp at level of 9 log CFU/g after incubation at pH 5 for 15 h (Coulin et al., 2006). Lactic acid bacteria have been encountered in a variety of plant materials such as olives, cocoa beans, sauerkraut and cassava (Peres et al., 2012). The reduction in bacterial number in later stages of the fermentation might be due to the low in pH of cassava pulp (Table 3.1).

Table 3.1 Bacterial counts (log CFU/g) and pH changes of cassava pulp sample at various times.

Types	Samples	Bacterial counts log (CFU/g, mL)	pH
Cassava pulp	0 day	7.31 ± 0.11^{b}	4.59 ± 0.04^{d}
	1st day	7.44 ± 0.08^{b}	4.47 ± 0.06^{d}
	3rd day	7.50 ± 0.05^{b}	4.23 ± 0.13^{c}
	5th day	7.45 ± 0.24^{b}	4.16 ± 0.08^{c}
	7th day	7.39 ± 0.23^{b}	3.97 ± 0.06^{b}
	14th day	6.42 ± 0.06^{a}	3.89 ± 0.08^{b}
	21th day	6.72 ± 0.13^{a}	3.80 ± 0.02^{b}
	28th day	6.33 ± 0.07^{a}	3.49 ± 0.04^{a}

Different superscript letters indicate significantly different results ($p<0.0.5$), n=6

3.4.2 Screening and selection of potential probiotic lactic acid bacteria

3.4.2.1 Determination of bile salt hydrolase (BSH) activity

In this study, the isolated *Lactobacillus* strains were evaluated for the cholesterol-lowering activity with BSH enzyme activity determination. It was found that out of 390 strains of lactic acid bacteria screened for BSH activity on MRS containing with 0.3% oxgall, 38 strains were positive as indicated by precipitation zones. BSH activity is shown on MRS with and without 0.3% oxgall. The strains that showed the largest precipitation zones were selected for further studies. Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease (Ramasamy et al., 2010). Although therapeutic drugs are availableto reduce this risk, they are still very expensive and always show the side effects of medicine. Several studies indicated that *Lactobacillus* species have been able to reduce cholesterol via several mechanisms (Kimoto et al., 2002; Ramasamy et al., 2010). The cholesterol-lowering effect of *Lactobacillus* species is by several means through bile salt hydrolase activity. BSH is the enzyme responsible for the bile salt deconjugation during enterohepatic circulation (Hernandez et al., 2005).

3.4.2.2 Determination acid and bile salt tolerance

Three isolates LAB that showed high BSH activities were selected. All isolates (3C2-10, 21C2-10 and 21C2-12) with acid and bile salt tolerance characteristics are shown in Table 3.2. Three selected isolates could survive exposure both 0.15 and 0.50% bile salt in MRS medium and pH level between 3-9 after incubation for 2 h. However, only 21C2-12 had survival rate 1.88 log CFU/mL at pH level 2. This was probably due to these strains were isolated from cassava pulp which fermented at pH between 3-4. However, the selected stains had potential to survive under bile and acid-base

environments. The selected strains tolerate bile and acidic conditions indicated that they could exist in stomach and bile intestine. Resistance against pH and bile salt are important criteria in its selection for probiotic used. The organisms taken orally have to face stresses from the host which begins in the stomach pH between 1.5 and 3.0, and in the upper intestine and colon contain high concentrations of bile. Thus, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v) which recommended as suitable concentration for *Lactobacillus* for human use (Goldin and Gorbach, 1992; Tambekar and Bhutada, 2010). It has been reported that the capacity of bacteria to resisting the toxicity of bile salt lead to better growth and higher assimilation of cholesterol (Parvez et al., 2005).

Table 3.2 Bile tolerance and pH dependent growth range for three isolated *Lactobacillus.*

Strains	Inoculum (log CFU/mL)	pH (log CFU/mL)							Bile salt	
		2	3	4	5	6	7	8	9	0.15%	0.50%
3C2-10	8.08±0.16	0.00	7.58 ±0.03	7.43 ±0.02	7.24 ±0.09	7.11 ±0.05	7.79 ±0.01	7.79 ±0.02	7.15 ±0.01	+	+
21C2-10	8.53±0.05	0.00	7.36 ±0.15	7.72 ±0.03	7.49 ±0.13	7.53 ±0.07	7.66 ±0.02	7.47 ±0.06	7.58 ±0.09	+	+
21C2-12	8.47±0.03	1.88 ±0.11	7.30 ±0.03	7.32 ±0.09	7.34 ±0.32	7.64 ±0.05	7.72 ±0.06	7.71 ±0.1	7.58 ±0.17	+	+

+, bacterial growth on MRS medium containing 0.15-0.50% bile salt .

3.4.2.3 Determination of antagonistic activity

The isolate 3C2-10 exhibited strong antimicrobial activities against 6 strains of enteric pathogens which indicated that they could be applied in the treatment and prevention of enteric infections (Table 3.3). Growth inhibition observation in this

study indicated that the assayed Lactobacilli produced antimicrobial products such as organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins inhibiting pathogens growth. Lactic acid bacteria have been reported to exert strong antagonistic activity against food contaminating microorganisms (Hernandez et al., 2005; Azadnia et al., 2011). Hernandez *et al*., (2005) noted that the presence of *L. plantarum* TF711 in the Tenerife cheese inhibited spoilage and pathogenic bacteria (*Bacillus cereus* ATCC10876, *Clostridium sporogenes* ATCC 3584, *Staphylococcus aureus* ATCC12600, *Shigella sonnei* E1 and *Klebsiella pneunoniac* E77). The antagonistic activity of probiotics has long been applied in food preservation. The antimicrobial substances produced by probiotics bacteria act for the improvement of the composition and normal microbiota activity in the intestine (Cizeikiene et al., 2013).

Table 3.3 Antagonistic activity of selected strains by agar well diffusion assay.

Strains	**Antagonistic activity**		
	3C2-10	**21C2-10**	**21C2-12**
Gram negative			
Enterobacter aerogenes bcc6719	+	-	-
Escherichia coli TISTR3436	+	-	-
Pseudomonus aeruginosa TISTR781	+	-	-
Gram positive			
Bacillus subtilis TISTR008	+	+	+
Bacillus cereus TISTR687	+	-	-
Staphylococcus aureus TISTR1466	+	-	-

-, no inhibition ; +, inhibition ; TISTR, Thailand Institute of Scientific and Technological Research.

3.4.2.4 Determination of cells adhesion and in cells surface hydrophobicity

Three selected strains (3C2-10, 21C2-10 and 21C20-12) were investigated for cells adhesion based on *in vitro* cell surface hydrophobicity and cells adhesion on Caco2 cell lines (Table 3.4). Strains 21C2-10 and 21C2-12 showed higher percent hydrophobicity than strains 3C2-10 and *L. plantalum* strain 1465 (commercial strain) in both hexadecane and iso-octane. Cell surface hydrophobicity related cell surface charge and hydrophobicity, which are nonspecific hydrophobic interactions, influenced the strength of adhesion (Duary et al., 2011; Monteagudo-Mera et al., 2012). The critically of cell surface is depends on bacterial cell surface hydrophobicity and

Table 3.4 Cells surface hydrophobicity (%) and cells adhesion of three selected strains.

Strains	**Cells surface hydrophobicity (%)**		**Cells Adhesion score (%)**
	Hexadecane	**Iso-octane**	
3C2-10	58.34±2.60[a]	46.16±1.37[a]	5.14±0.35[a]
21C2-10	92.62±2.83[c]	76.14±1.93[c]	13.23±1.08[c]
21C2-12	87.40±1.04[b]	65.65±1.73[b]	8.78±0.90[b]
L.plantarum strain 1465 (commercial strain)	56.22±2.39[a]	48.27±2.70[a]	3.94±0.88[a]

Different superscript letters are significantly different ($p<0.05$), n=3

bacterial surface charges (van der Waals an electrostatic forces) which are consequences of the chemical composition of the bacterial surfaces including contact between both bacterial cell membrane and interacting surfaces (Duary et al., 2011). Positive cell adhesion to hydrocarbons are considered to be hydrophobic and negative are hydrophilic. The quantitative binding of three selected strains were also tested on Caco-2 cell lines (Table 3.4). Strain 21C2-10 (13.23±1.08) was the highest adhesive strains based on adhesion score ($p<0.05$). Strain 21C2-12 (8.78±0.90) showed significant difference with commercial strain (*L. plantalum* strain 1465) while strain 3C2-10 (5.14± 0.35) was no significant difference with commercial strain. Hence, cells surface hydrophobicity is associated with cells adhesion (Caco-2 cell lines) ability. Caco-2 cells are human intestinal epithelium derived cancer cells (colonic adenocarcinomas) and it has been suggested to indicate adhesion ability of Lactobacilli (Monteagudo-Mera et al., 2012). Kotzamanidis *et al*., (2010) reported that cell-surface hydrophobicity and Caco-2 cells line can considerably influence the strength of adhesion that used for the adherence potential of probiotic strains assessment and could be valuable for establishing in human clinical studies.

3.4.2.5 Determination of prebiotic activity

Prebiotic activity scores showed in Figure 3.1. The selected strains (3C2-10, 21C2-10, 21C2-12 and OC4-4) grew on a prebiotic substrates as inulin, Lactulose and FOS but not on cassava pulp and cellulose. While *Lactobacillus plantarum* LAB1465 grew on all prebiotic substrates tested, fructooligosaccharide (FOS) had the highest prebiotic activity scores in all strains (3C2-10, 21C2-10, 21C2-12, OC4-4 and LAB1465). In contrast, cassava pulp and cellulose showed the lowest prebiotic scores. Prebiotic activity indicated the ability to stimulate of bacterial growth relative to non-prebiotic substrates, such as glucose (Huebner et al., 2007). Therefore, a positive

prebiotic activity score showed good growth as well as glucose by probiotic strains. *Lactobacillus* spp. have the ability to metabolize prebiotic carbohydrate to formation of lactic, acetic and other short chain organic acids (Huebner et al., 2008). The organic acids are may be associated with antagonistic activity against intestinal competitors (Wang, 2009).

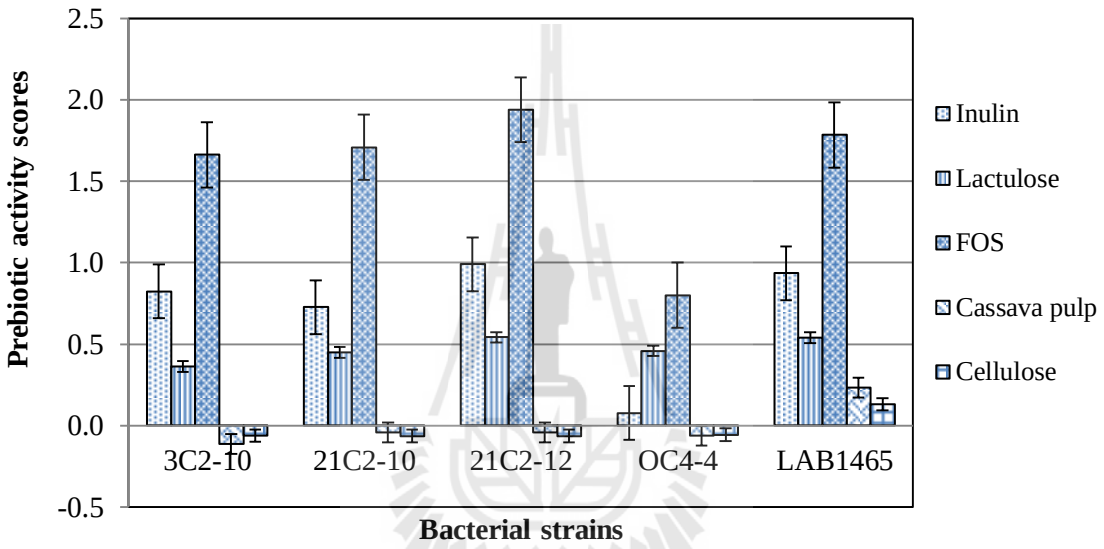

Figure 3.1 Prebiotic score of *Lactobacillus* strains (3C2-10, 21C2-10, 21C2-12, OC4-4 and *Lactobacillus plantarum* LAB 1465).

3.4.3 Cholesterol-lowering activity

Cholesterol-lowering activity in active cells broth of selected strains and control is shown in Table 3.5. The cholesterol reduction of 3C2-10, 21C2-10, 21C2-12, 0C4-4, 1C4-3 and 1C4-11 were 24, 24, 18, 6, 8 and 2 µg/mL respectively. However, cholesterol reduction of dead cell of these strains could not decrease cholesterol concentration in culture broth. Percentage of residual cholesterol reduction compared between active cells, cell pellets and dead cell broth is also illustrated in Figure 3.2.

The greater reduction of cholesterol by active cells over the dead cells also corresponded to the growth of cells (Liong and Shah, 2005). The active cells of 3 strains (3C2-10 21C2-10 and 21C2-12) significantly exhibited the high ability to decrease cholesterol from broth ($p<0.05$). Three isolates, strains 3C2-10, 21C2-10 and 21C2-12, were evaluated for the cholesterol-lowering activity via BSH enzyme activity and the capability of bacterial cell to remove cholesterol from culture broth. These results suggested that the BSH ability supported the mechanism for the *in vitro* lowering of cholesterol of the cells (Kumar et al., 2012). On the contrary, 0C4-4, 1C4-3 and 1C4-11 did not show BSH activity but still had the ability to reduce cholesterol from culture broth by active cells but at much lower levels which ranged from 2-8 µg/mL. This result might be due to the acid produced from natural lactic acid fermentation of Lactobacilli strains. The precipitation of cholesterol in supernatant appeared to be related to the deconjugation of bile salts and their subsequent precipitation at low pH which range from 4.63-5.05. The pH of culture broth decreased due to the organic acid production by the bacteria (Sirilun et al., 2010). Bile acids are less soluble and are more likely to precipitate at pH lower than 5.5 (Nguyen et al., 2007; Li, 2012). In this study the active cells removed more cholesterol than dead cells and cell pellets (Figure 3.2). In addition, the ability of cholesterol-lowering activity of the strains without BSH activity might be due to some cholesterol was bound to the bacterial cellular surface (Figure 3.2 and 3.3). This result was agreed with the study of Kimoto *et al.*, (2002) and Kumar *et al.*, (2012) who reported that some Lactobacilli could remove cholesterol from suspension in culture broth during growth. Cholesterol was also removed by incorporation of probiotics into the cellular membranes during growth (Huey-Shi et al., 2010; Ooi and Liong, 2010; Li, 2012). Lye *et al.*, (2010) also further evaluated this mechanism by determining the possible locations of the

incorporated cholesterol within the membrane phospholipid bilayer of probiotic cells. Enrichment of cholesterol was found in the regions of the phospholipid tails, upper phospholipids, and polar heads of the cellular membrane phospholipid bilayer in cells that were grown in the presence of cholesterol compared to the control cells, indicating incorporation of cholesterol in those regions (Huey-Shi et al., 2010).

The incorporation of cholesterol into the cellular membrane increased the concentration of saturated and unsaturated fatty acids, leading to increased membrane strength and subsequently higher cellular resistance toward lysis (Huey-Shi et al., 2010).

Table 3.5 Change of cholesterol content in broth of active cell during 20 h incubation of culture.

Strains	**Cholesterol lowering content (µg/mL)**	**pH**	**BSH activity**
Control	DL	5.85 ± 0.05^{d}	-
3C2-10	24.00 ± 5.47^{b}	4.63 ± 0.08^{a}	+
21C2-10	24.00 ± 5.48^{b}	4.58 ± 0.07^{a}	+
21C2-12	18.00 ± 4.47^{b}	4.79 ± 0.02^{b}	+
0C4-4	6.00 ± 5.48^{a}	4.96 ± 0.11^{c}	-
1C4-3	8.00 ± 4.42^{a}	5.05 ± 0.06^{c}	-
1C4-11	2.00 ± 4.40^{a}	4.94 ± 0.04^{c}	-

Different superscript letters are significantly different ($p<0.0.5$), n=5, DL=detection limit.

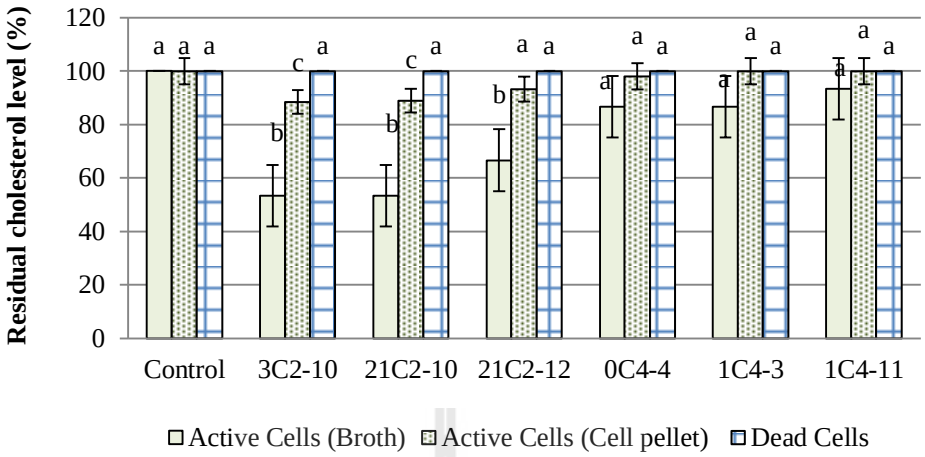

Figure 3.2 Residual cholesterol reduction level (%) of active cells (broth), active cells (cells pellet) and dead cells during 20 h incubation of culture. The error bars indicated the standard deviation (SD) between individual trials and different superscript letters are significantly different ($p<0.05$), n=3.

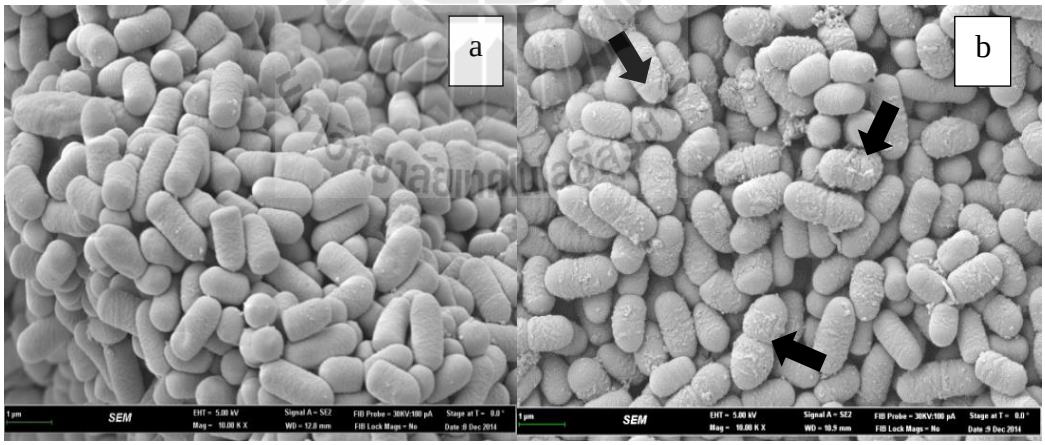

Figure 3.3 Scanning electron micrograph of strain 21C2-10 (a) media without cholesterol (b) broth supplemented with cholesterol incubated at 37^{o}C for 20 h.

3.4.4 Identification of potential probiotic lactic acid bacteria

Carbohydrate fermentation of 3 isolates was tested using API50 CHL kit as shown in Table 3.6. Based on carbohydrate fermentation pattern analysis, 3C2-10 was identified as *L. plantarum* while 21C2-10 and 21C2-12 were identified as *L. pentosus* and *L. brevis* respectively. The results agreed with previous studies that the kit is not accurate enough to identify the species level in some cases (Khunajakr et al., 2008). *L. plantarum* has been frequently found in plant materials including cassava (Peres et al., 2012). The species *L. plantarum, L. pentosus,* and *L. paraplantarum* were genotypically related with highly similar phenotypes (Khunajakr et al., 2008; Mangell et al., 2012). Therefore, the isolates were confirmed by partial 16S rDNA sequence analysis (Table 3.7). Corresponding partial sequences of 3 selected strains, the results showed that sequences similar to their corresponding strains from GenBank. 3C2-10, 21C2-10 and 21C2-12 were identified as *L. plantarum, L. acidophilus* and *L. fermentum* respectively, at 99% similarity,. As shown in Table 3.7, strains 21C2-10 and 21C2-12 were classified as *L. pentosus* and *L. brevis*, respectively by API 50CHL kit which was re-identified as *L. fermentum* and *L. plantarum*, respectively based on partial 16S rDNA sequence analysis. While 3C2-10 was identified as *L. plantarum* in both methods, the API50 CHL system failed to identify some *Lactobacillus* species. Various species of lactic acid bacteria have been reported as being predominant in plant materials especially cassava pulp (Peres et al., 2012). However, different dominant species have been reported even though the isolated strains originated from the same sources. This difference might be due to the various factors such as type of cassava and other environmental factors.

Table 3.6 Morphological, physiological and biochemical characteristics of the selected strains.

	Strains		
	3C2-10	**21C2-10**	**21C2-12**
Gram stain	G+	G+	G+
Spores formation	-	-	-
Catalase activity	-	-	-
Motility	+	+	+
Facultative anaerobic	+	+	+
Sugar Fermentation			
L-Arabinose	+	+	+
Ribose	+	+	+
D-Xylose	-	+	+
Galactose	+	+	+
Fructose	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	-
Cellobiose	+	+	-
Maltose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Saacharose	+	+	+
Trehalose	+	+	-
Raffinose	+	+	-

-, negative ; +, positive

Table 3.7 Identification of selected strains by API 50 CHL kit and sequence analysis of 16S rRNA gene.

Strains	Identification with API 50 CHL		Identification based on 16S rRNA gene		
	Identification	% identity	Identification	% similarity	NCBI Accession no.
3C2-10	*L. plantarum*	99.9	*L. plantarum* strain WG27	99	K5779104
21C2-10	*L. pentosus*	93.9	*L. acidophilus*	99	JQ350808
21C2-12	*L. brevis*	99.4	*L. fermentum* strain LG1	99	KC348395

3.5 Conclusions

Probiotic lactic acid bacteria were isolated from cassava pulp being silaged at various times under anaerobic conditions at 37°C. The isolates were selected based on their BSH activity and also identified by the combination of morphological, biochemical characteristics and partial 16S rRNA sequence. The isolates were identified as *L. plantarum* (3C2-10), *L. acidophilus* (21C2-10) and *L. fermentum* (21C2-12). The selected isolates could tolerate bile salt at the concentration at 0.15 and 0.50% (w/v). Three selected isolates (3C2-10, 21C2-10 and 21C2-12) also resisted pH level between 3-9 during 2 h. Only 21C2-12 could survive at pH level 2. The strain 3C2-10 exhibited strong antimicrobial activities against *Enterobacter aerogenes* bcc6719, *Bacillus subtilis* TISTR008, *Escherichia coli* TISTR3436, *Bacillus cereus* TISTR687, *Pseudomonus aeruginosa* TISTR781 and *Staphylococcus aureus* TISTR1466. Three strains (3C2-10, 21C2-10 and 21C2-12) exhibited the bile salt hydrolase (BSH) activity and cholesterol-

lowering properties with the reduction rate 18-24 µg/mL by active cells, but not in dead cell. The 3 isolates showed strength of cell adhesion and had the ability to metabolized FOS, lactulose and inulin. Three selected strains were identified as *L. plantarum*, *L. acidophilus* and *L. fermentum* found to be the predominant species in cassava pulp and had a tendency to be used in food products for cholesterol-lowering property.

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CHAPTER IV

PROTECTIVE EFFECTS OF CEREAL AND CASSAVA PULP EXTRACTS ON THE SURVIVAL OF POTENTIALLY PROBIOTIC LACTIC ACID BACTERIA UNDER SIMULATED GASTRO-INTESTINAL TRACT CONDITIONS

4.1 Abstract

The effect of cereal and cassava pulp extracts that are used as food carriers for delivering potential probiotic lactic acid bacteria (LAB) through the gastrointestinal tract (GI) system was evaluated. Viability of three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) incubated with cassava pulp, brown rice and rice bran were not significant different. The growth of three *Lactobacillus* strains were supported by all tested three extracts and reached total count between 8.50-9.69 log CFU/mL within 24-48 h, when compared to log CFU/mL in MRS-medium. The influence of incubation with three extracts as cassava pulp, brown rice and rice bran to the viability of three *Lactobacillus* strains was studied. The condition is in simulated gastric juice at pH 2.0 for 2 h and in intestinal juice at pH 7.4.0 for 4 h. The viability of three *Lactobacillus* strains were improved by approximately 7 log CFU/mL in the presence of cassava pulp, brown rice and rice bran with the condition pH 2 for 1 h incubation time. In addition, all strains were significantly decreased in their cell population when incubated without cereal and cassava pulp extracts at the condition at pH 1-2 for 2 h

incubation period. However when using only cassava pulp extract under pH 2 for 2 h, survival cells were not found. After sequential incubation in simulated gastric at pH 2.0 for 1 h following with intestinal juices at pH 7.4 for 4 h, three *Lactobacillus* strains showed the number of survival cells at level of 5.27-8.83 log CFU/mL. These results indicated that cassava pulp, brown rice and rice bran extracts exhibited a significant protective effect on the viability of three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) under gastrointestinal conditions.

Keywords: *Lactobacillus*;,Cassava pulp, Brown rice; Rice bran; Cereals; gastrointestinal tract conditions.

4.2 Introduction

Due to the tremendous increase in public concern over health issues and the diversification of food products, the functional food industry needs to be explored the novel processes. Basic nutritional functions are the concept of functional food which has positive effects on host health as modulators of blood pressure, and it also reduces the risk of chronic disease or serum cholesterol levels (Michida et al., 2006). Probiotic microorganisms, especially lactic acid bacteria (LAB), are generally considered as functional foods, based on the presumption that culture viability is a reasonable measure of probiotic activity. Delivery foods or nutritive supplements should contain probiotic populations of 10^7cells/mL at the time of consumption in order to provide health benefits for the host (Nase et al., 2001; Martins et al., 2013).

Lactobacilli strains are among the LAB strains that are the most widely used as oral probiotics to improve the function of the gastrointestinal (GI) tract and health of

the host. *Lactobacillus* strains have generally been recognized as having safe (GRAS) status for applications in foods, and numerous species are used world-wide in food fermentations (Bourdichon et al., 2012). Many probiotics are sensitive to the low pH of the stomach and high bile salt concentration in the proximal intestine, causing survival during passage of the upper GI tract to be an important concern that live and functional probiotic microorganisms reach their target sites in the distal portion of the GI tract (Kent and Doherty, 2014).

Prebiotics are indigestible polysaccharide compounds that pass undigested through the upper gastrointestinal tract and stimulate the growth or activity of one or more species of colonic bacteria as probiotic by acting as substrate (Broekaert et al., 2011; Slavin, 2013). These compounds are present in various cereal grains which are known as prebiotics (Michida et al., 2006).

Rice (*Oryza sativa*) and cassava (*Manihot esculenta*) are the most important cereal crops in the world. Thailand is a major producer of cassava and rice (Chauynarong et al., 2015). Rice is one of the basic grains of people's daily diet throughout the world especially in Asia. Rice is an important source of carbohydrates, protein, iron, calcium, thiamine, riboflavin and niacin (Bienvenido, 1993). Brown rice consists of the whole grain including the hull and bran around the kernel. It is a highly nutritious grain containing magnesium, phosphorus, selenium, thiamin, niacin and vitamin B6, and manganese (Huang et al., 2016). Rice bran is the outer layer that surrounds the endosperm of whole grain brown rice. It contains tocopherols, tocotrienols, anthocyanins, polyphenols, g-oryzanol, enzymes, polyunsaturated fatty acids and resistant starch (Premsuda et al., 2011). While, cassava pulp is a fibrous by-product of the cassava processing industry, it also contains about 50-60% starch on a

dry weight basis, 1.5-5% protein, 0.1-4% fat and 10–15% fibers, which are composed mainly of cellulose and other non-starch polysaccharides (Montagnac et al., 2009). In previous studies, cereals were evaluated as being good substrates for the growth of probiotic strains and enhancing acid and bile tolerance (Charalampopoulos et al., 2002; Blaiotta et al., 2013). In addition, they improve gut health and immunity, protect against harmful microorganisms, and reduce infection severity. In addition, they show anti-hypertensive, anti-carcinogenic benefit and obesity prevention. Thus cereal food is considered to be a functional food.

The purpose of this research was to select new functional cereal foods, which could be use as the substrate for gastrointestinal improvement and to examine the effects of indigestible cereal and cassava pulp extracts on the viability of *Lactobacillus* strain during passage of simulated upper gastro-intestinal tract conditions.

4.3 Materials and methods

4.3.1 Preparation of cells

Lactobacillus spp. (3C2-10, 21C2-10 and 21C2-12) was originally isolated from cassava pulp from a factory in Thailand (Nawong et al., 2013). Fresh cell suspensions were prepared for each experiment and enumerated by drop plating on De Man Rogosa Sharpe (MRS) agar. Plates were incubated anaerobically at 37°C for two days.

Lactobacillus strains were obtained in MRS broth incubated anaerobically at 37°C for 24 h. The cells were harvested with centrifugation at 5000 rpm for 10 min, and the cell pellets were washed twice with peptone saline (PS, 1 g/L peptone; Oxoid, 8.5 g/L NaCl; Fisher Scientific, Canada) and resuspended in the same solution.

4.3.2 Preparation of cereal and cassava pulp

Cassava pulp, brown rice and rice bran were used for the preparation of cereal extracts following the methods of Michida et al., 2006. The grains were ground with a Falling Number hammer mill (Retsch GmbH & Co. KG, Haan, Germany) using a sieve size of 0.08 mm. Grain flours of 10 g were mixed with 90 mL of distilled water and heated at 80^{o}C for 20 min and sterilized at 121^{o}C for 15 min. Cereal extracts were centrifuged at 5000 rpm for 10 min and sterilized at 121^{o}C for 15 min and were kept in refrigerator until use.

4.3.3 Preparation of simulated gastric and intestinal juice

Simulated gastric juice (SGJ) was prepared by dissolving pepsin (pepsin A from porcine stomach mucosa P-7000, Sigma) in saline water (SW, 0.2% NaCl, w/v) to a final concentration of 0.3 g/L and adjusted to pH 2.0 with concentrated HCl. The mixture was sterilized and filtering through a membrane filter (0.2 µm; Corning® Corning Incorporated, Germany).

Simulated intestinal juice (SIJ) was prepared based on the method of Huang and Adams (2004). Pancreatin (P-8096, Sigma) and bile salts (Oxgall, Difco, Fisher Scientific) were suspended in phosphate buffer (PB, 0.02 M) to final concentrations of 1 g/L and 4.5 g/L, respectively. The mixture was adjusted to a pH 7.4 with 0.1 mol/L NaOH and filter sterilized (Huang and Adams, 2004). Fresh simulated gastric and intestinal juices were prepared fresh just before test.

4.3.4 Measurement of gastric and bile tolerance

Washed cell suspension of *Lactobacillus* spp. (3C2-10, 21C2-10 and 21C2-12) were prepared using the method of Blaiotta et al. (2013). Aliquots (0.2 mL) of the cell suspension were transferred to a 2.0 mL capacity Eppendorf tube, mixed with 0.3 mL

of sterile saline (0.5%, w/v) or cereal extracts (5% w/w), and finally mixed with 1.0 mL of simulated gastric (pH 2.0) or bile juice (pH 7.4) (Blaiotta et al., 2013). The mixture was then incubated at 37 ^{o}C. In gastric and bile challenge tests for the total viable count were measured after 0, 30, 60 and 120 min of incubation.

4.3.5 Viable cell count

The spot plating method was used to determine cells viability following Blaiotta et al. (2013). Ten microliters were dropped onto MRS agar plates and incubated at 37^{o}C for 48 h in anaerobic condition. The individual colonies were counted and calculated as log 10 colony-forming units per mL.

4.3.6 Chemical analysis

The soluble free amino nitrogen (FAN) content was determined by the ninhydrin colorimetric method (Hagen and Schwarz, 2000), using a glycine solution (2 mg/l) as control. Total soluble sugar concentrations were determined by the phenol-sulphuric acid method (Dubois et al., 1956). Reducing sugar concentrations were estimated by the 3,5-dinitrosalicylic acid method (Miller, 1959) using glucose as standard sugar solutions.

4.3.7 Statistical analysis

Four independent experiments with duplicate samples were conducted for each treatment. Results were statistically analyzed where appropriate by Analysis of Variance (ANOVA) using SPSS 16.0 software package for Windows.

4.4 Results and Discussion

4.4.1 Chemical composition of cassava pulp and cereal extracts

The chemical analysis of cassava pulp and cereal extracts without microorganisms is shown in Table 4.1. The concentrations of total sugars of cassava pulp and all cereal extracts and MRS broth were higher than that of reducing sugar. The higher concentrations of total sugars than reducing sugar could be related to the sucrose or other soluble oligosaccharides (Charalampopoulos et al., 2002). Brown rice flour extract showed higher total sugar and reducing sugar concentrations than cassava pulp and rice bran. Rice bran in the outer layer is a by-product of the rice milling process which is a rich source of protein but not starch (Gao et al., 2008; Chotiko and Sathivel, 2014), therefore, the total sugar and reducing sugar are less than brown rice. As a result, free amino nitrogen is higher in rice bran than cassava pulp and brown rice extracts. The composition of the cereal extracts was important for gastric tolerance because they contained water-soluble sugar and free amino nitrogen (Michida et al., 2006). The physical and chemical properties of cassava and cereals are considered as buffering capacity and pH of food carries (Blaiotta et al., 2013). Moreover, cassava pulp and cereals are also soluble fiber, oligosaccharides and resistant starch which might be considered as prebiotic concept (Rivera-Espinoza and Gallardo-Navarro, 2010).

Table 4.1 Chemical composition of the sterile cassava pulp and cereal extract suspensions.

	MRS-broth	**Cassava pulp**	**Brown rice**	**Rice bran**
pH	**5.85 ± 0.06^{a}**	**6.75 ± 0.16^{a}**	**6.54 ± 0.09^{a}**	**6.59 ± 0.13^{a}**
Total sugars (ug/mL)	1191.35 ± 0.28^{a}	42.82 ± 0.17^{c}	103.45 ± 0.22^{b}	14.72 ± 0.15^{d}
Reducing sugars (ug/mL)	18.40 ± 0.18^{a}	5.36 ± 0.07^{c}	8.96 ± 0.13^{b}	2.46 ± 0.06^{d}
Free amino nitrogen(mg/L)	64.77 ± 2.25^{a}	2.10 ± 0.05^{d}	6.09 ± 0.03^{c}	11.71 ±0.13^{b}

Row with superscript different letters are significantly different ($p<0.05$), n=3

4.4.2 Effects of cassava pulp, brown rice and rice bran on cell viability

The viability of three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) with cassava pulp, cereal extracts and MRS broth during 0, 24 and 48 h are shown in Figure 4.1. The viability of the three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) did not change in growth significantly ($p<0.01$) after the addition of the cereal extracts (cassava pulp, rice brown and rice bran) during 24 and 48 h of incubation which were approximately 8.43-8.96 log cycles in all the treatments tested. Excluding, the three *Lactobacillus* strains for which the number increased from 8 to 10 log CFU/mL during 24 h in the MRS medium at 37^{o}C. Even after 48 h incubation, the viable population of three *Lactobacillus* strain decreased slightly with approximately 0.76-1.08 log cycles in the MRS medium. The high cells viability observed for the three *Lactobacillus* strains in the MRS medium but not in cassava pulp, brown rice and rice bran at 24 and 48 h incubation. Charalampopoulos et al, (2003) suggested that FAN concentrations ranging from 13 to 47 mg/L did not improve cell viability and had no obvious positive effect on the cell stability of *L. plantarum*. However, the cassava pulp and cereal extract had no effect on the growth of the *Lactobacillus* strains and not toxic to the bacterial cells. These results demonstrated that cassava pulp and all cereal extracts were suitable food carriers that supported the survival of potentially probiotic *Lactobacillus* strains.

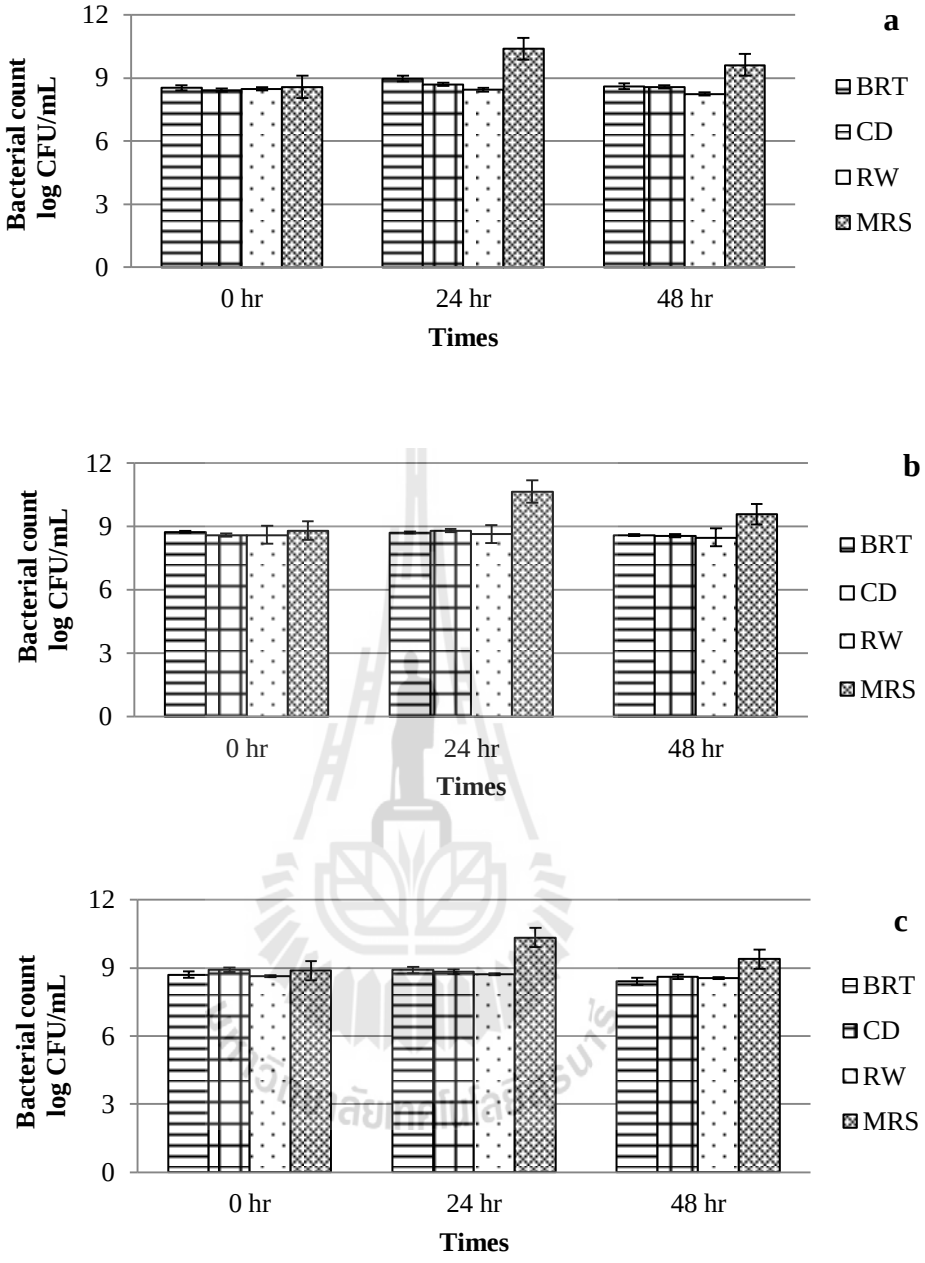

Figure 4. 1 Growth of three *Lactobacillus* strains in cassava pulp and cereal extract (BRT: brown rice, CD: cassava pulp and RW: rice bran), a) 3C2-10, b) 21C2-10 and c) 21C2-12. Average (n=5) are shown with error bars.

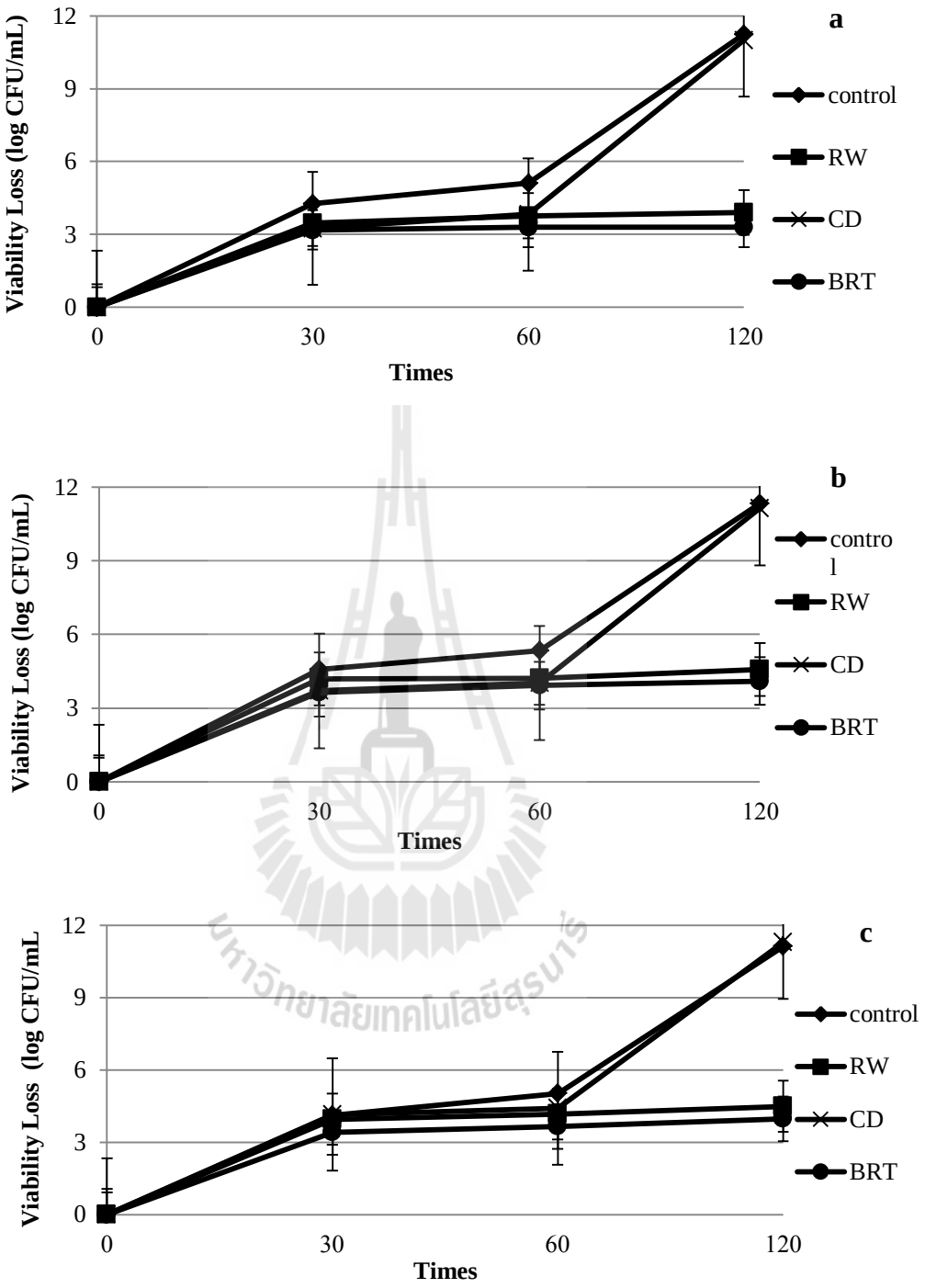

Figure 4.2 The effect of cassava pulp and cereal extracts on SGJ during exposure for 120 min in rice bran (RW), cassava pulp (CD) and brown rice (BRT) of three *Lactobacillus* strains a) 3C2-10, b) 21C2-10 and c) 21C2-12. Average (n=5) are shown with error bars.

4.4.3 Effect of cassava pulp and cereal extracts on survival of LAB in simulated gastric juice

The effect of cassava pulp and cereal extracts on survival of three *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12) within the simulated gastric juice (SGJ) during 120 min is shown in Figure 4.2. The cell reduction of *Lactobacillus* strains 3C2-10, 21C2-10 and 21C2-12 without cassava pulp and cereal extracts (control) was approximately at 4, 5 and 11 log (CFU/mL) after exposure to SGJ for 30, 60 and 120 min, respectively. At pH 2, cassava pulp and control were not able to significantly ($p<0.05$) reduce the viability of all tested strains after incubation time 120 min. Free cells (control) and cassava pulp extract were not effective for protecting cells in the SGJ. The undissociated form of low pH was assumed to be the most toxic form for bacterial cells (McDonald et al., 1990). A high survival rate was observed for all cereal extracts after exposure to SGJ for 30 and 60 min respectivey. The best protective effect was obtained in brown rice and rice bran where the reduction in growth was approximately 3 log cycles after 120 min. Rice bran and brown rice improved the survival rate of three *Lactobacillus* strains in comparison with the survival rate observed for free cells following exposure to SGJ (pH 2) for up to 120 min at 37^{o}C and were not significantly different from each other ($p>0.05$). However, the simulation of gastric juice *in vitro* probably overestimate viability losses relative to the scenario *in vivo* because food components might temporarily elevate the gastric pH (Borza et al., 2010). Consequently, a strong correlation of the final survival rate of the *Lactobacillus* strains within the cassava pulp and cereal extracts in SGJ for 60 and 120 min were expected.

Probiotic bacteria must pass through the stomach in the area where the pH can be as low as 2 to 3 (Nase et al., 2001). Cereal and cassava pulp extracts could protect bacterial cells under acid conditions which help *Lactobacillus* pass through the small intestine and colon and thus contribute to intestinal microflora balancing. However, the time from entrancing to being released from stomach was estimated to be approximately 90-120 min for solid food and 60 min for liquid food, with further digestive processes requiring a longer residence time (Kong and Singh, 2008). Therefore, it is essential that potential probiotic Lactobacilli should be able to survive at pH between 2 and 3 for up to 120 min. Acid tolerance ability of the bacteria is another option might prove to be useful. For applications in host environmental conditions and food systems, the acid tolerance of Lactobacilli should be considered.

4.4.4 Effects of cassava pulp and cereal extracts to survival of LAB with simulate gastric and intestinal juice

The survival of three *Lactobacillus* strains (with the extracts) and free cells (no added the extracts; control) after 30 min in SGJ followed by 60 and 240 min incubation in SIJ with bile (pH 7.4) is shown in Figure 4.3. For free cells, the viable cells were reduced by 4 log units after the initial exposure to SGJ for 30 min and decreased by up to 6 log units after incubation in SIJ for 240 min (Figure 4.3). While cereal extracts, rice bran, cassava pulp and brown rice co-incubation supported the survival of all three *Lactobacillus* strains after sequential exposure to SGJ for 30 min followed by 240 min in SIJ. The viability of the three *Lactobacillus* strains was reduced by approximately 3.62-4.33 log units after incubated in SIJ for 240 min. There were significant changes ($p<0.05$) with the survival rate for all *Lactobacillus* strains (3C2-10, 21C2-10 and 21C2-12), incubation with all extracts. The results

demonstrated that cassava pulp and cereal extracts affected on the survival rate in the upper gastrointestinal tract. This research confirms some previous reports on the survival of *Lactobacillus plantarum* co-incubated with cereal extracts such as barley and wheat extracts which had a synergistic effect on the gastrointestinal tract (Charalampopoulos et al., 2003; Michida et al., 2006).

The effect of cassava pulp and cereal extract on the survival of probiotic bacteria under gastrointestinal tract conditions were reported by several studies (Charalampopoulos et al., 2003; Michida et al., 2006; Broekaert et al., 2011; Blaiotta et al., 2013; Aumiller et al., 2015). Generally microorganisms are inhibited at low pH when entering the gastrointestinal tract in the undissociated form and then they dissociate afterwards. Cytoplasm is collapsed by the proton motive force by causing acidification, resulting in inhibition of nutrient transport (McDonald et al., 1990; Charalampopoulos et al., 2002) . Cereals also contain water-soluble fibers, oligosaccharides and resistant starch (Blaiotta et al., 2013) which have a good buffering capacity for food carrier. However, the results showed that cereals were significant factors in improving the viability of probiotic lactic acid bacteria during exposure to pH 2 of the simulated gastric transit for 2 h and could be delivered to the simulated intestine pH 7.4 for 4 h, This was probably due to an alternative source as a part of a buffered food delivery system.

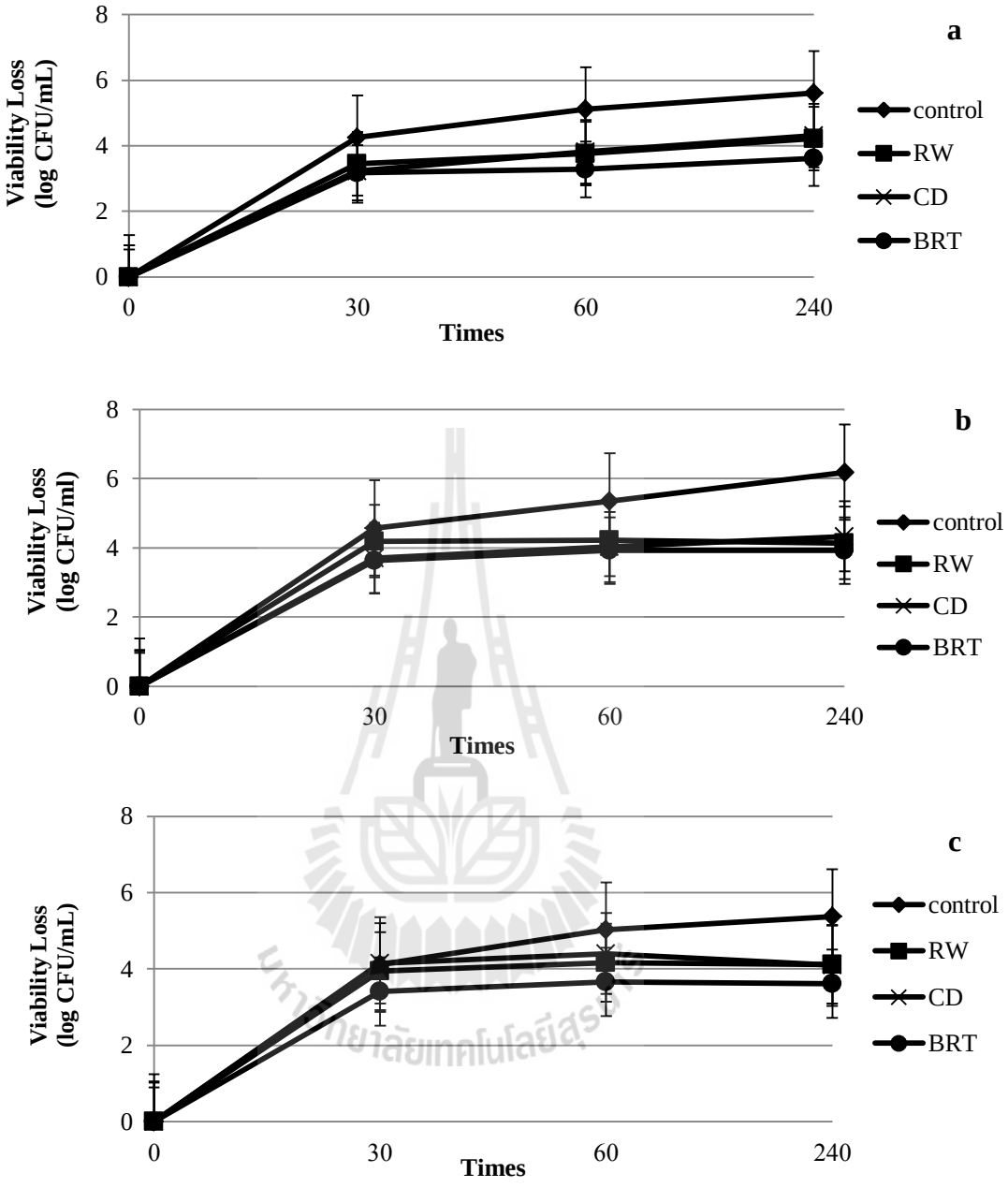

Figure 4.3 The effect of cassava pulp and cereal extracts during sequential exposure to SGJ and SIJ at 37°C in rice bran (RW), cassava pulp (CD) and brown rice (BRT) of three *Lactobacillus* strains a) 3C2-10, b) 21C-10 and c) 21C-12. Average (n=5) are shown with error bars.

4.5 Conclusions

In conclusion, cassava pulp and cereal extracts significantly improved the simulated gastric and bile tolerance of three strains of *Lactobacillus* sp. (3C2-10, 21C2-10 and 21C2-12). Brown rice extract showed a greater improvement in survival compared to rice bran and cassava pulp extract, probably because of its composition in terms of water-soluble sugar and free amino nitrogen content. Positive effect of the presence of cassava pulp and cereal extracts in this study (brown rice, cassava pulp and rice bran) showed these to be candidate food matrices which can be used as probiotic vehicles for enhancing the transit tolerance of three strains of *Lactobacillus* sp.

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CHAPTER V

ENTRAPMENT IN FOOD-GRADE TRANSGLUTAMINASE CROSS-LINKED GELATIN-MALTODEXTRIN MICROSPHERES PROTECTS *LACTOBACILLUS* SPP. DURING EXPOSURE TO SIMULATED GASTRO-INTESTINAL JUICES

5.1 Abstract

Dietary intake of probiotic bacteria has been shown to impart health effects, however, maintaining viable cells in foods and during passage of the adverse conditions in the upper gastro-intestinal tract is often a problem. The objective of this research was to develop and characterize novel food-grade phase-separated gelatin-maltodextrin (G-MD) microspheres, where the gelatin was cross-linked with transglutaminase (TGase), to determine if encapsulated probiotic lactic acid bacteria were protected during exposure to simulated upper gastro-intestinal tract conditions. The stability, size, structure and protective ability of G-MD microspheres as a function of different TGase concentrations and gelatin bloom strengths were tested. The G-MD microspheres made with gelatin A 300 bloom and a TGase concentration of 10 U/g prevented pepsin-induced degradation of the microspheres in simulated gastric juice (pH 2.0, 2 h, 37°C), resulting in significantly ($P<0.05$) higher numbers of survivors due to the buffering effect of intact microspheres (average diameter 46 μm).

After sequential incubation in simulated gastric (1 h) and intestinal juices (pH 7.4, 4 h, 37°C), survivor levels of each of the three encapsulated *Lactobacillus* sp. (3C2-10, 21C2-10 and 21C2-12) were reduced by 0.2-1 log (CFU/g) as compared to 3-4 log(CFU/g) for the free non-encapsulated cells. The microspheres containing the *Lactobacillus* sp. remained physically intact during 40 day of refrigerated storage. This study presents a novel protein based microencapsulation method, which using all food-grade ingredients protects probiotic lactic acid bacteria during exposure to adverse environmental conditions.

Keywords: Gelatin, maltodextrin, transglutaminase, microencapsulation, probiotics, lactic acid bacteria, simulated gastro-intestinal juices, functional foods.

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5.2 Introduction

Lactobacillus spp. are among the lactic acid bacteria (LAB) strains that are most widely used as oral probiotics to improve the function of the gastrointestinal (GI) tract and health of the host (Anandharaj and Sivasankari, 2014). *Lactobacillus* strains are generally recognized as being safe (GRAS) status for applications in foods, and numerous species are used world-wide in food fermentations (Bourdichon et al., 2012). Many probiotics are sensitive to the low pH of the stomach and high bile salt concentration in the proximal intestine, causing survival during passage of the upper GI tract to be an important concern in ensuring that live and functional probiotic microorganisms reach their target sites in the distal portion of the GI tract (Kent and Doherty, 2014). Delivery foods or nutritive supplements should contain probiotic populations of at least 10^6 live microorganisms per gram or milliliter at the time of

consumption in order to provide health benefits to the host (Sohail et al., 2013; Kent and Doherty, 2014).

Microencapsulation is a promising technology that may be useful for the oral delivery of live probiotic bacteria (Guerin et al., 2003; Heidebach et al., 2009; Malmo et al., 2013). In this technique, the probiotic bacterial payload is essentially packed into microspheres composed of one or many different types of biologically compatible and non-toxic bio-polymers derived from natural sources such as seaweed (alginate, carrageenan), plants (starch, gum arabic, soy protein and pea protein), bacteria (gellan, xanthan), and animals (milk, gelatin, whey protein) using techniques such direct gelation, emulsion and complex coacervation (Dong et al., 2013; Bosnea et al., 2014; Eratte et al., 2015; Wang et al., 2015). Ideally, the microspheres should be cheap to make from readily available bio-polymers and designed to be semipermeable, spherical, strong and capable of retaining the bacterial cells until the controlled release under desired specific conditions. Also, the microspheres size should be small (less than <100 µm in diameter) so as not to affect the sensory attributes negatively when incorporated into foods. If properly designed, microencapsulation has the potential to enhance protection of probiotic cells during exposure to adverse conditions in food and the GI-tract (Sohail et al., 2013; De Prisco and Mauriello, 2016).

Gelatin is an approved and commonly used ingredient in the food and pharmaceutical industry with potential application in microencapsulation formulations (Rokka and Rantamäki, 2010). However, microspheres formed by gelatin alone are physically weak and prone to rapid enzymatic degradation by digestive enzymes. To overcome this problem, use of biocompatible cross-linkers such as genipin has been shown to stabilize gelatin based microspheres, thus enchancing the protection of entrapped bifidobacteria during exposure to simulated gastro-intestinal conditions

(Annan et al., 2007). However, cross-linking with genipin resulted in the microspheres turning dark blue thus limiting their potential use in food products.

Presence of maltodextrin increases the yield of live and functional probiotic LAB cells after exposure to thermal stress during spray drying (Reddy et al., 2009). Enhanced protection during simulated upper GI tract transits was also observed for the probiotic *Bifidobacterium adolescentis* encapsulated in genipin cross-linked mixed gelatin-maltodextrin (G-MD) microspheres in comparison to survival in gelatin-only microspheres (Borza et al., 2010).

The promise of the G-MD matrix for microencapsulation led us to search for alternative non-toxic and colourless gelatin cross-linkers. Transglutaminase (TGase, EC. 2.3.2.13), which catalyses an acyl-transfer reaction between the carboxyamide group of peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the amino group of lysine residues to form an ε-(γ-glutamyl) lysine isopeptide bond (Yung et al., 2007), appeared to fulfill these requirements. The GRAS status of TGase has meant that the enzyme is widely used in different food products to improve functional properties such as hydration, stability of gels and emulsions or foaming properties of soybean proteins (DeJong and Koppelman, 2002; Cho et al., 2003; Borza et al., 2010; Pavunc et al., 2011).

The aim of the study was to develop a method for producing TGase cross-linked G-MD microspheres using food grade ingredients and to investigate the structure and stability of these microspheres depending on the TGase concentration and gelatin gel (bloom) strength as well as their ability to release and protect entrapped probiotic *Lactobacillus* spp. originally isolated from Cassava pulp, during exposure to the adverse environment found in the upper segments of the GI tract. We report here that encapsulation in translucent TGase cross-linked G-MD microspheres significantly

($P<0.05$) improved the survival of three cassava derived LAB in simulated gastric and intestinal juices in comparison to the survival obtained for non-encapsulated cells.

5.3 Materials and methods

5.3.1 Preparation of cells for microencapsulation

Lactobacillus spp. strains 3C2-10, 21C2-10 and 21C2-12 were originally isolated from cassava pulp from a factory in Thailand using deMan Rogosa Sharpe (MRS) agar (Oxoid, UK) and characterized using the API 50 CHL test kits according to the manufacturer's instructions (BioMérieux, Marcy-I'Etoile, France) (Nawong et al., 2013).

Aliquots of frozen stock cultures (1 mL, stored at -80°C in MRS broth [CM0359, Oxoid, Fisher Scientific, Nepean, ON, Canada] with 30% (v/v) glycerol, [Fisher Scientific]) of the lactic acid bacterial strains were inoculated in 100 mL MRS-cys broth, which consisted of MRS broth supplemented with 0.5 g/L of filter sterilized L-cysteine (Fisher Scientific) and 10 g/L maltodextrin (MD, Dextrose Equivalent (DE) 1, Star Dry® 1, Decatur, IL, USA). The cultures were incubated at 37°C for 24 h to obtain cell densities of about 9-10 log colony forming units per mL (log(CFU/mL)). Cells was harvested by centrifugation at 3,578 × *g* (Universal 32R, Hettich Centrifuges, Concord, ON, Canada) for 10 min at 4°C and after discarding the supernatant of spent culture broth, the cell pellet was resuspended in peptone saline (PS, 1 g/L peptone; Oxoid, 8.5 g/L NaCl; Fisher Scientific, Canada) and centrifuged again under the same conditions. Fresh cell suspensions were prepared for each experiment and enumerated by spread plating on MRS-cys agar, which consisted of MRS agar with filter sterilized L-cysteine (0.5 g/L). Plates were incubated anaerobically at 37°C for two days.

5.3.2 Preparation of TGase cross-linked G-MD microspheres with encapsulated lactic acid bacteria

The original G-MD microencapsulation method (Borza et al., 2010) was modified to test the feasibility of using TGase (Transglutaminase Activa TI, Ajinomoto, France) as the cross-linker of the gelatin phase. Briefly, the microspheres were prepared using porcine gelatin of various bloom (i.e., gel) strengths, i.e., gelatin A 60 (type A, 60 Bloom, Sigma, St. Louis, MO, USA), gelatin A 175 (type A, 175 Bloom, Sigma) and gelatin A 300 (type A, 300 Bloom, Sigma). Separate stock solutions of gelatin (24% w/w) and maltodextrin (24% w/w) were prepared in 0.5% (w/v) NaCl at 45˚C and stirred for 20 min. One gram of washed bacterial suspension, prepared as described in section 2.1 and containing 9-10 log(CFU), was added to the maltodextrin stock solution to obtain a cell to solution ratio of 1:15. Next, gelatin was mixed with maltodextrin at a ratio of 2:1. Variable TGase concentrations of 5, 10 or 15 units per g (U/g) of gelatin were depending on the experiment added to the gelatin-maltodextrin-cell aqueous mixture (pH 6.5-7), which was then emulsified in tempered oil (45˚C, aqueous to oil phase volume ratio of 1:5) containing 0.5% (w/w) Span 85 (Sigma, USA) using a Caframo Real Torque Digital Stirrer (Caframo Ltd., Wiarton, ON, Canada) to stir the mixture at a constant speed (900 rpm). The emulsion was left stirring overnight (around 18 h) at room temperature (22-23˚C) to allow for the completion of the TGase cross-linking reaction.

A washing solution (8.5 g/L NaCl, 5 g/L Tween 80 (Sigma) in distilled water) was added at a ratio of 1:1 to the aqueous gelatin-maltodextrin phase and the microspheres were harvested with two subsequent washings followed by gentle centrifugation (500×*g*, 1 min, room temperature) to remove the oil phase, and stored at 4˚C until further analysis.

5.3.3 Enumeration of microencapsulated bacteria

Entrapped bacteria in the TGase cross-linked G-MD microspheres were released by enzymatic digestion of gelatin using collagenase (*Clostridium histolyticum* Type IA, C-9891, Sigma). An aliquot (0.5 g) of the microsphere slurry was added to 4.5 g of the 40 U/mL collagenase solution containing 0.5 mM $CaCl_2$ (Fisher Scientific, Canada) and pH adjusted to 7.4. After incubation at 37°C for 1 h, the released cells were spread plated on MRS-cys agar plates, which were enumerated following anaerobic incubation for two days at 37°C with the number of viable encapsulated bacteria reported as CFU per g of the microsphere slurry. The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as:

$$EY(\%) = \frac{N}{N_0} \times 100$$

where N is the number of viable entrapped cells released from the microspheres, and N_o is the number of free cells added to the biopolymer mix during the production of the microspheres.

5.3.4 Particle size measurement

The particle size of microspheres was determined by measuring the diameter of 600 microspheres at x 1000 magnification using an optical microscope (Eclipse 80i, Nikon Canada Inc., Mississauga, ON, Canada) fitted with a calibrated micrometer scale. The mean diameters of microspheres were calculated and presented with standard deviations (n-1).

5.3.5 Microscopy to determine the structure of the gelatin-maltodextrin microspheres

The structure and thickness of the gelatin phase in the TGase cross-linked G-MD microspheres were examined by confocal laser scanning microscopy (CLSM, Zeiss LSM 510 Laser Scanning Confocal Imaging System, Carl Zeiss, Jena, Germany). The gelatin in the phase separated microspheres was labelled by adding 0.01% (w/v) Rhodamine B isothiocyanate (RITC-B, Sigma-Aldrich, Canada) directly to the biopolymer mixtures used for microsphere preparation. RITC-B stained microspheres were immobilized in glycerol and scanned (magnification × 1000) using the Z-series mode at a distance of 1.0-2.0 µm between layers. Confocal microscopy images were acquired using an argon laser emitting at 543 nm, a bypass (BP) filter detecting at 560-615 nm, a long pass (LP) filter detecting at 505 nm and analyzed using the LSM 510 software (Carl Zeiss).

5.3.6 Survival of free and encapsulated *Lactobacillus* spp. in simulated gastric juice

Simulated gastric juice (SGJ) was prepared by dissolving pepsin (pepsin A from porcine stomach mucosa P-7000, Sigma) in saline water (SW, 0.2% NaCl, w/v) to a final concentration of 0.3 g/L and adjusting the pH to 2.0 with concentrated HCl. The mixture was sterilized by passing through a membrane filter (0.2 µm, Corning® Corning Incorporated, Germany). Fresh SGJ was prepared for use on the same day.

Washed cell suspensions of *Lactobacillus* sp. (0.5 mL) or 0.5 g of microspheres with entrapped bacteria were added to 4.5 mL of tempered (37°C) SGJ, mixed well by gentle vortexing for 10 s and incubated for 30, 60 and 120 min at 37°C. Entrapped cells were after each set time interval released from microspheres. Surviving

encapsulated and free (non-encapsulated) bacteria were enumerated on MRS-cys agar as described above in section 5.2.3.

To test the effect of TGase concentration and gelatin bloom strength on survival of encapsulated *Lactobacillus* sp. 3C2-10 in comparison to non-encapsulated cells during exposure to SGJ, microspheres were made with (1) gelatin A 300 and varying TGase concentrations (5, 10 or 15 U/g), and (2) different gelatins (60, 175 or 300 bloom strength) and 10 U/g TGase, respectively. The survival of free and encapsulated *Lactobacillus* spp. strains 3C2-10, 21C2-10 and 21C2-12 in G-MD microspheres made with gelatin A 300 and 15 U/g TGase was also compared.

5.3.7 Survival of free and encapsulated *Lactobacillus* spp. after sequential incubation in simulated gastric and intestinal juices

Concentrated simulated intestinal juice (SIJ) was freshly prepared based on a previously published method (Huang and Adams, 2004), where pancreatin (P-8096, Sigma) and bile salts (Oxgall, Difco, Fisher Scientific) were suspended in phosphate buffer (PB, 0.02 M) to final concentrations of 8 g/L and 36 g/L, respectively, followed by adjustment of pH to 7.4 with 0.1 mol/L NaOH and filter sterilization.

Using the same microsphere formulations as described above in section 2.6, washed *Lactobacillus* spp. suspensions (0.5 mL) or 0.5 g of microspheres with encapsulated bacteria were added to 4.5 mL of tempered (37°C) SGJ, mixed well by gentle vortexing for 10 s and incubated for 60 min at 37°C. Concentrated SIJ tempered at 37°C was then added (1.25 mL) and pH adjusted to 7.4 with 0.1 M NaOH. The final volume of the mixture was made up to 10 mL with PB to yield final SIJ concentrations of 1 g/L and 4.5 g/L of pancreatin and bile salts, respectively, and incubated for 240 min at 37°C. Surviving bacteria after set times of sequential

incubation (60 min in SGJ, 60, 120 , 180 and 240 min in SIJ) were enumerated as described in section 5.3.3.

5.3.8 Stability of microspheres during exposure to simulated gastric juice

The stability of microspheres during exposure to SGJ was assessed by observing the structural integrity using an optical microscope (magnification×1000). Microscopy was performed on microspheres prepared with 10 U/g TGase and gelatin A 300, gelatin A 175 or gelatin A 60, during their exposure to SGJ for 0 (control), 30 and 60 min at 37°C.

5.3.9 Viability of *Lactobacillus* strains in microspheres during refrigerated storage time

The viability of microspheres during refrigerated storage was evaluated by enumerated as described in section 5.3.3. The viability of bacterial cells was monitored after the microencapsulation process and also at the end of storage period.

5.3.10 Statistical analysis

Four independent experiments with duplicate samples were conducted for each treatment (n=8). Results were statistically analyzed where appropriate by one-way Analysis of Variance (ANOVA) using SPSS 16.0 software package for Windows (IBM Corp., Armonk, NY, USA) with the Tukey post hoc test at the 5% significance levels. The size of the microspheres (diameters) was not normally distributed according to the Shapiro-Wilk test, leading to the use of the non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

5.4 Results

5.4.1 Size and encapsulation yields of gelatin-maltodextrin microspheres made with different gelatins and transglutaminase concentrations

The novel microencapsulation method successfully entrapped the bacteria in TGase cross-linked G-MD microspheres, which were translucent in colour and contained levels of the probiotic bacteria of 10^{10} CFU/g. Encapsulation yields for *Lactobacillus* sp. 3C2-10 ranged from 89.6 to 92.3% and were similar regardless of the gelatin bloom strength or TGase concentration (Table 5.1).

Sizing of G-MD microspheres made with entrapped *Lactobacillus* sp. 3C2-10 and a TGase concentration of 10 U/g but varying gelatin bloom strength showed that the mean diameter of microspheres made with gelatin A 300 bloom (46 μm) was significantly ($P<0.05$) smaller than the mean diameter (60 μm) obtained for microspheres made with gelatin A 60 and 175 (Table 5.1). When varying the TGase concentration, it was observed that G-MD microspheres made with gelatin A 300 and 5 or 10 U/g TGase were not significantly ($P>0.05$) different from each other (48 vs. 47 μm, respectively) but significantly ($P<0.05$) smaller than microspheres made with 15 U/g TGase, which had mean diameters of 52 μm (Table 5.1).

Table 5.1 Size and encapsulation yields of *Lactobacillus* sp. 3C2-10 in transglutaminase cross-linked gelatin-maltodextrin microspheres at different bloom strength gelatin and various transglutaminase concentrations.

Microsphere Gelatin Bloom	**TGase Concentration (U/g)**	**Size (µm)**	**Encapsulation Yield (%)**
60	10	60.2±14.8[c]	92.29±3.81[a]
175	10	60.4±16.6[c]	90.09±4.54[a]
300	10	45.9±15.9[a]	91.40±4.43[a]
300	5	47.9±15.7[a]	90.46±6.00[a]
300	10	45.9±15.9[a]	89.97±5.64[a]
300	15	52.2±13.4[b]	89.55±5.59[a]

Different superscript letters are significantly different ($p<0.05$), n=600

5.4.2 Phase contrast and confocal laser scanning microscopy (CLSM) of gelatin-maltodextrin microspheres made with TGase

Optical CLSM images taken through the equator of microspheres showed the separation of the biopolymers, where the RITC-B labelled gelatin phase, regardless of the TGase concentration, formed a uniform outer membrane (16-20 µm thick) on the outside of an inner core that consisted of the unlabelled maltodextrin (Figures 1b, 1d and 1f). The images obtained from phase contrast microscopy similarly revealed the presence of two phases (Figures 1a, 1c and 1e). The micrographs also demonstrated that cells of the entrapped *Lactobacillus* sp. 3C2-10 were predominantly located at the interphase between the gelatin and maltodextrin phases with some occurring in the

gelatin outer membrane (Figure 5.1).

5.4.3 Survival of free and encapsulated Lactobacillus spp. during exposure to simulated gastric juice

Encapsulation in TGase cross-linked G-MD microspheres significantly ($P<0.05$) improved survival of the *Lactobacillus* strains in comparison to the survival observed for non-encapsulated free cells following exposure to SGJ (pH 2) for up to 120 min at 37^{o}C (Figure 5.2). The viability of free cells of *Lactobacillus* sp. 3C2-10 was reduced by approximately 4 log(CFU/g) after exposure to SGJ for 30 min at pH 2, while significantly ($p<0.05$) lower reductions of 0.5 log(CFU/g) after 30 min, as numbers of cells dropped from 10^{9} CFU/g to $10^{8.5}$ CFU/g in the SGJ, were observed for cells entrapped in G-MD microspheres made with gelatin A 300 and 5, 10 or 15 U/g of TGase (Figure 5.2a). Statistical analysis revealed that the reduction in viable counts after 60 and 120 min exposure to SGJ was significantly ($p<0.05$) lower for bacteria entrapped in microspheres cross-linked by 10 and 15 U/g of TGase as use of higher bloom strength gelatin A 300 significantly ($p<0.05$) enhanced bacterial survival during exposure to simulated SGJ with final reductions of 0.44 log(CFU/g) after 120 min (Figure 5.2b). In contrast, cells entrapped in microspheres made with gelatin A 60 and 175 exhibited reductions of 0.94 and 1.32 log (CFU/g), respectively.

The significant ($p<0.001$) increase in the number of survivors was the same for all *Lactobacillus* sp. strains (3C2-10, 21C2-10 and 21C2-12) following microencapsulation in G-MD microspheres made with gelatin A 300 and 10 U/g TGase, relative to the survival of non-encapsulated cells of the same strain (Figure 5.2c).

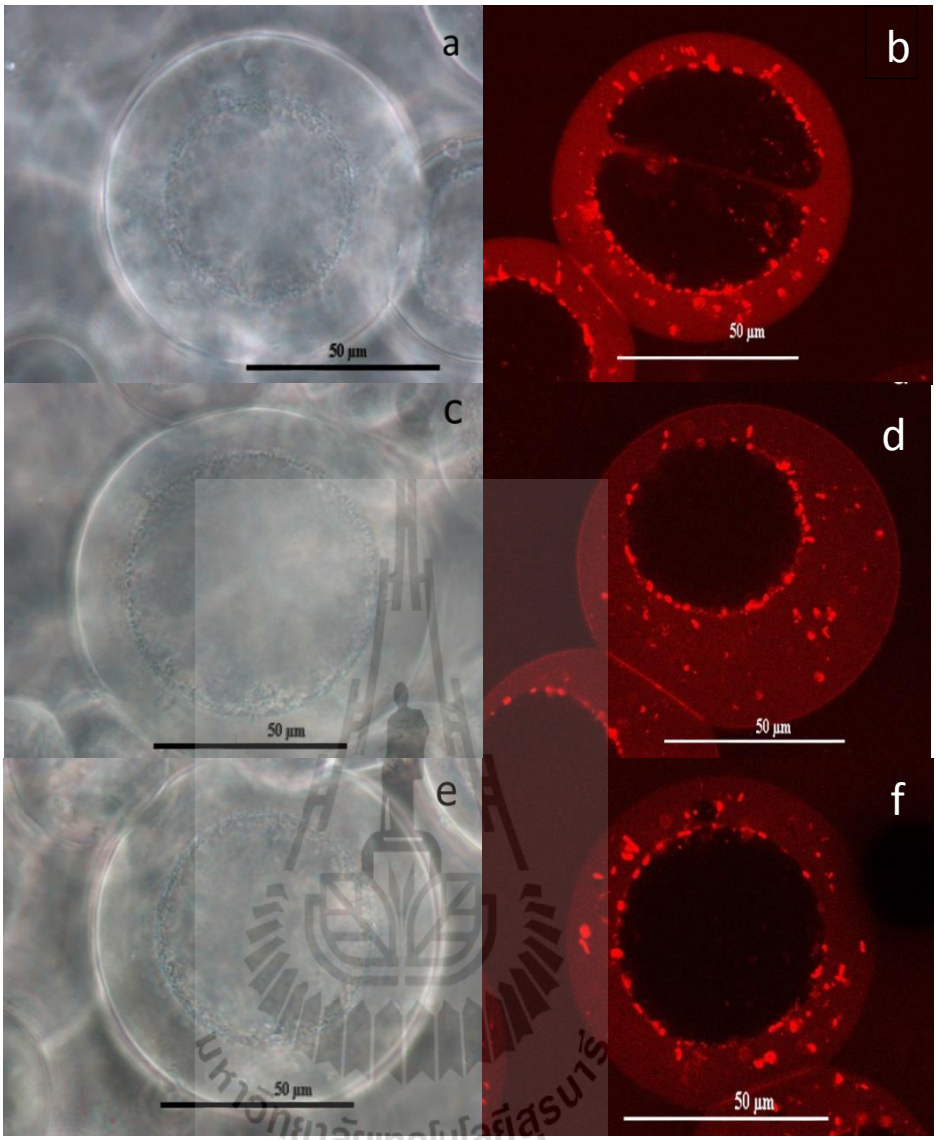

Figure 5.1 Microstructure as observed by phase contrast (panels a, c and e) and confocal laser scanning (panels b, d and f) microscopy of unstained and rhodamine B isothiocyanate (RITC-B) stained, respectively, TGase cross-linked G-MD microspheres made with gelatin A 300 bloom and TGase concentrations of 5 (panels a and b), 10 (panels c and d) and 15 U/g (panels e and f). Entrapped *Lactobacillus* strain 3C2-10 cells appear bright red in the confocal laser scanning images.

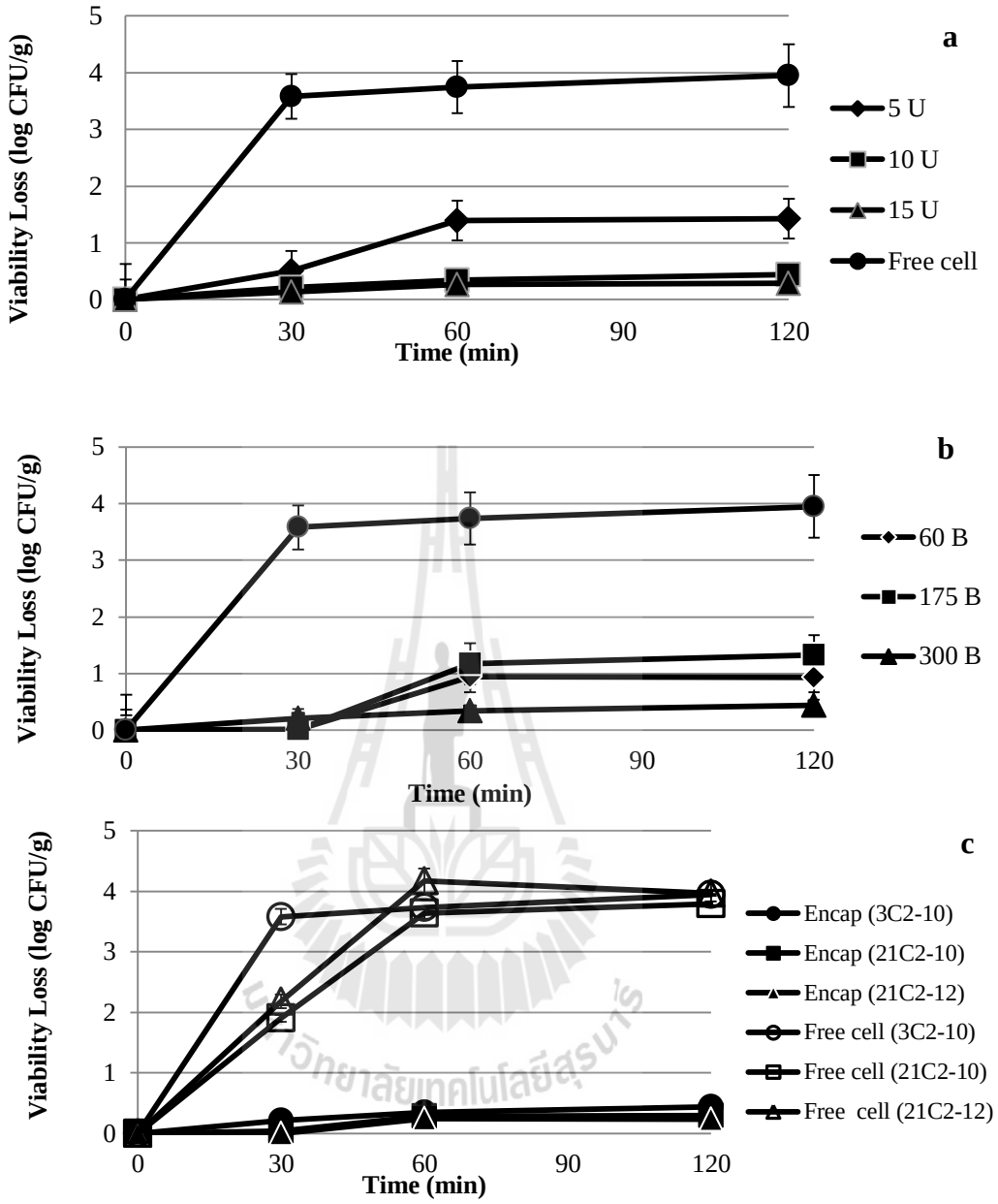

Figure 5.2 Survival of entrapped *Lactobacillus* cells following exposure to SGJ (37°C) and microencapsulation a) of *Lactobacillus* strain 3C2-10 in G-MD microspheres made with gelatin A 300 and TGase concentrations of 5,10 and 15 U/g, b) of *Lactobacillus* strain 3C2-10 in G-MD microspheres made with gelatin A 60, 175 and 300, and 10 U/g TGase, and c) of three different *Lactobacillus* spp. in G-MD microspheres formulated with gelatin A 300 and 10 U/g TGase. Average log(CFU/g) reductions (n=4) are shown with error bars.

compared to 5 U/g TGase with final losses of 0.3-0.5 log(CFU/g) vs. 1.4 log(CFU/g), respectively, and 4 log(CFU/g) for free cells (Figure 5.2a).

5.4.4 Viability of free and encapsulated cells of lactic acid bacteria during sequential incubation in simulated gastric and intestinal juices

Encapsulated *Lactobacillus* spp. survived sequential exposure to SGJ for 60 min followed by 120, 180 and 240 min incubation in SIJ with bile (pH 7.4) significantly ($p<0.05$) better than free cells (Figure 5.3). For free cells, viable numbers were reduced by 4 orders of magnitude after the initial incubation in SGJ for 60 min to remain at this reduced level (Figure 5.3). Microencapsulation dramatically increased the survival by at least 3 log units regardless of the encapsulation method or strain. Exposure to SIJ caused no further decrease in viable counts. In agreement with the results obtained for survival of SGJ alone (Figure 5.2b), the formulation made with gelatin A 300 yielded the best survival (Figure 5.3b) while at that bloom strength use of various concentrations of TGase (5, 10 and 15 U/g) made no significant difference ($p>0.05$) (Figure 5.3a). Microencapsulation supported equal survival of all three tested *Lactobacillus* sp. strains after sequential exposure to SGJ and SIJ (Figure 5.3c).

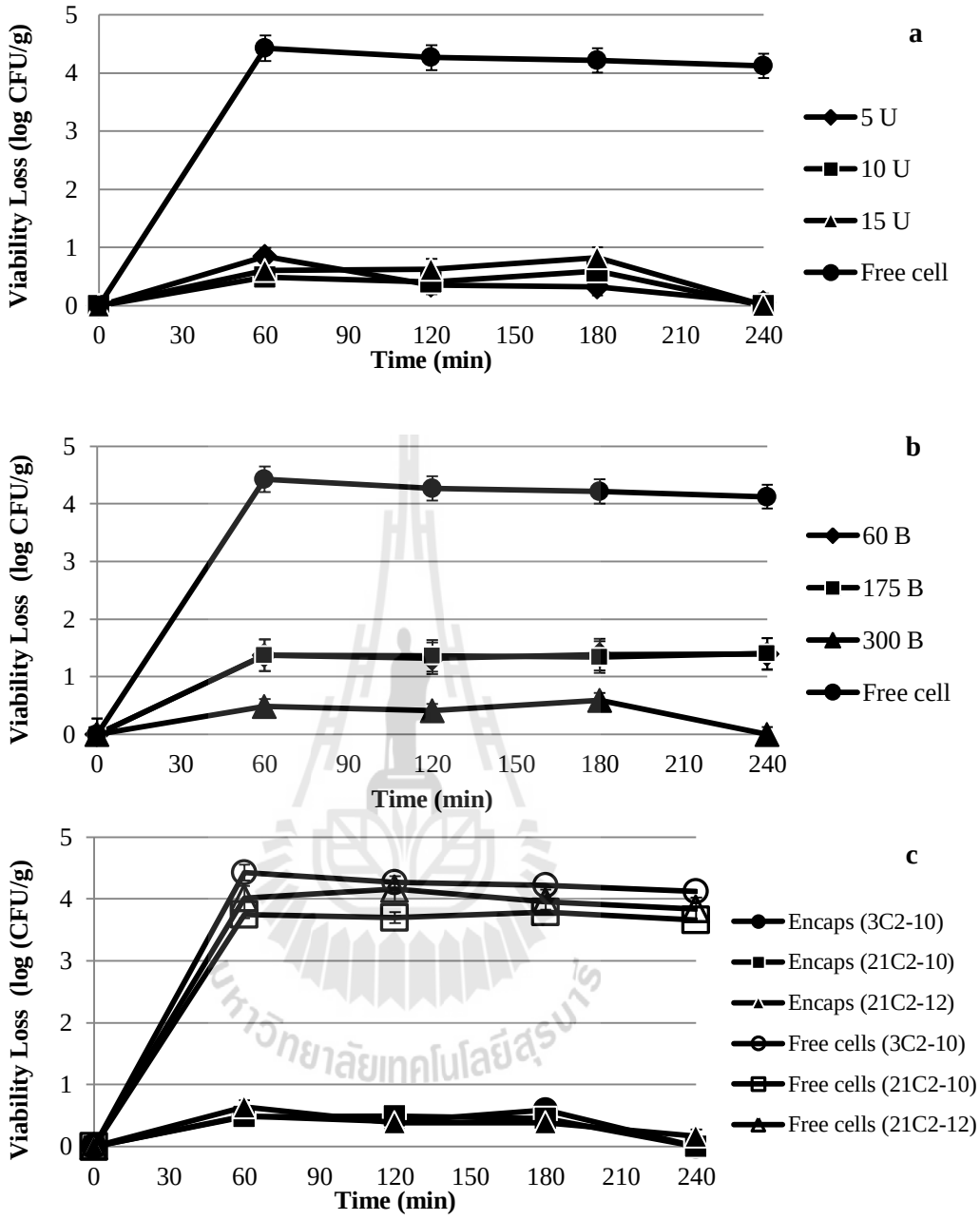

Figure 5.3 Survival of free and microencapsulated lactic acid bacteria during sequential exposure to SGJ (first hour) and SIJ (subsequent 3 hours) at 37°C in a) G-MD microspheres made with gelatin A 300, TGase concentrations of 5, 10 and 15 U/g and *Lactobacillus* sp. 3C2-10 , b) G-MD microspheres made with gelatin A of 60, 175 and 300 bloom strength and *Lactobacillus* sp. 3C2-10, and c) G- MD microspheres made with gelatin A 300 and 15 U/g TGase and

Lactobacillus sp. strains 3C2-10, 21C2-10 or 21C2-12. Average log(CFU/g) reductions (n=4) are shown with error bars.

5.4.5 Stability of transglutaminase cross-linked gelatin-maltodextrin microspheres during exposure to simulated gastric and intestinal juices

Phase contrast microscope images revealed that microspheres made with gelatin A 60 completely disintegrated after just 30 min in SGJ (Figures 4a, b and c) while those made with gelatin A 175 maintained the structure partially after 30 min to more fully disintegrate after 60 min (Figures 4d, e and f) before reaching the SIJ. In contrast, use of gelatin A 300 resulted in microspheres that remained structurally intact after exposure to SGJ for 60 min, thus indicating that the higher gel strength imparted resistance to degradation by pepsin in the acidic SGJ and helped maintaining the viability of *Lactobacillus* cells entrapped in the gelatin matrix (Figures 4g, h and i). The entrapped lactobacilli became released from the gelatin A 300 microspheres as they disintegrated during exposure to SIJ for 1 h (data not shown).

5.4.6 Viability of *Lactobacillus* strains in microspheres during refrigerated storage time

The viablity of encapsulated *Lactobacillus* stains was significantly ($p<0.05$) better than that of free cells during refrigerated storage period lasting for up to 57 days (Figure 5.5). The initial cell of free cells contained 7-8 log units decreased to 2 log units day 6 and all free cells dropped belows the detection limit after 7 day of refrigerated storage (Figure 5.5). For encapsulated *Lactobacillus* strains, viable numbers were consisnt along refrigerated storage period (Figure 5.5b). The formulation of gelatin A 300 at various concentrations of TGase (5, 10 and 15 U/g) remained constant and revealed no significant differences ($p>0.05$) of viable cells

(Figure 5.5a). Microencapsulation provided viablity of all three *Lactobacillus* strains at refrigerated storage time.

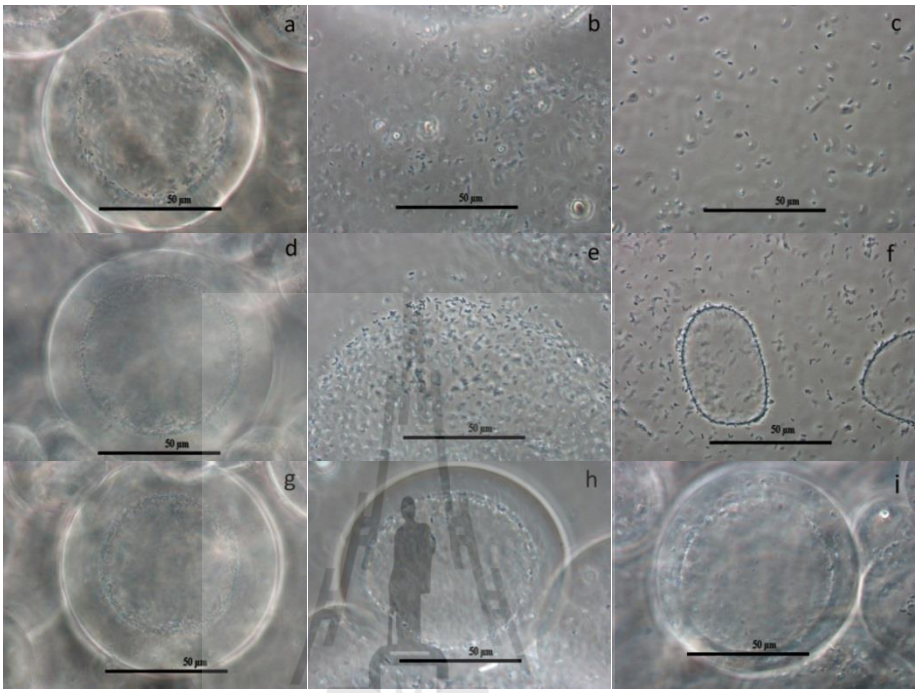

Figure 5.4 Phase contrast microscopy images (× 1,000 magnification) showing the stability of TGase cross-linked G-MD microspheres with entrapped *Lactobacillus* sp. 3C2-10 during exposure to SGJ at 37°C. Microspheres made with TGase (10 U/g) made with TGase (10 U/g) and gelatin A 60 bloom (a, b, and c), gelatin A 175 bloom (d, e and f) and gelatin A 300 bloom (g, h and i) were observe after 0, 30 and 60 min of exposure to SGJ.

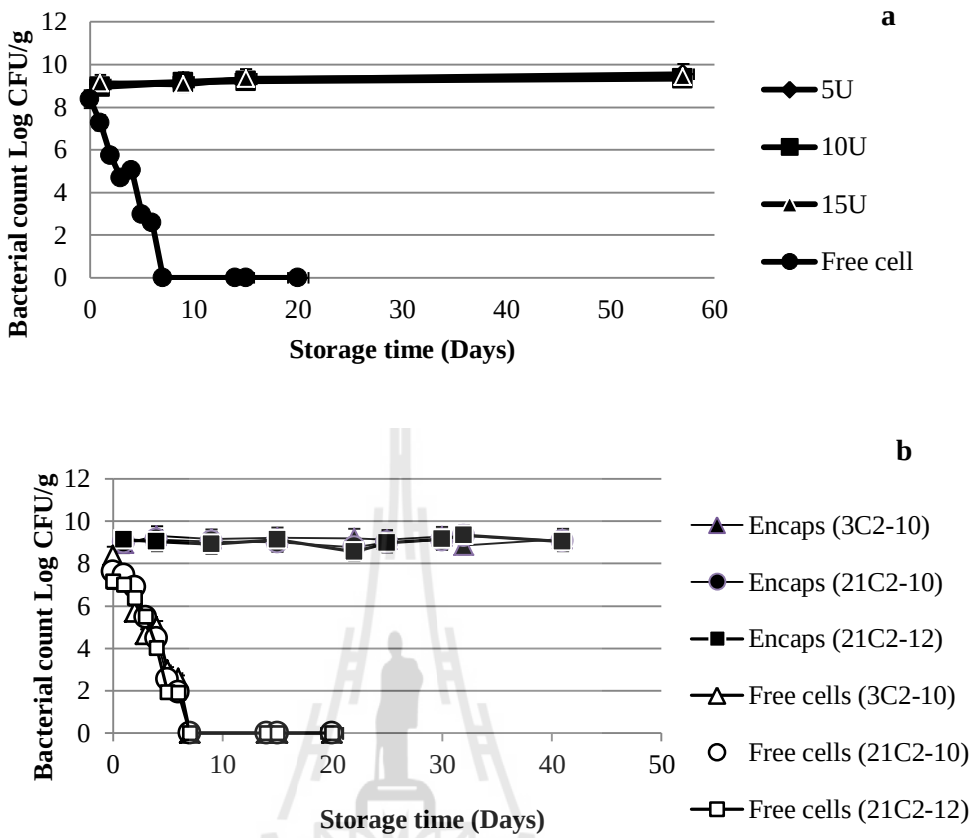

Figure 5.5 Viability of free and microencapsulated lactic acid bacteria during refrigerated storage (4°C) in a) G-MD microspheres made with gelatin A 300, TGase concentrations of 5, 10 and 15 U/g and *Lactobacillus* sp. 3C2-10 and b) G-MD microspheres made with gelatin A 300 and 15 U/g TGase and *Lactobacillus* sp. strains 3C2-10, 21C2-10 or 21C2-12. Average log (CFU/g) reductions (n=3) are shown with error bars.

5.5 Discussion

5.5.1 Size, encapsulation yields and structure of microspheres

The size of the G-MD microspheres depended on the TGase concentration and bloom strength of the gelatin (Table 5.1). TGase functions by catalyzing the formation of covalent N ε-(γ-glutamyl) lysine amide bonds between individual gelatin strands to form a permanent network of polypeptides (Chen et al., 2003; Cho et al., 2003). The high level of TGase activity over a wide range of temperatures, ion concentrations and pH values enables to use of the enzyme in a wide variety of gel formation conditions and cell encapsulation techniques (Yung et al., 2007). The highest concentration of TGase (15 U/g), which was used in the present study, increased the average microsphere diameter slightly possibly due to the quicker conversion of the gelatin solution into a cross-linked gel network ensuring minimal changes in shape and size of microspheres (Heidebach et al., 2009). Increasing the bloom strength from 60 and 175 to 300 decreased the microsphere diameter, which was contrary to previous observations for genipin cross-linked gelatin microspheres (Annan et al., 2007). The change in the cross-linking agent from genipin to the TGase enzyme may account for this difference, where in the latter case the less viscous droplets in the gelatin A 60 and A 175 may have had time to coalesce before the enzyme acted to set the microsphere gel. Importantly, the microsphere size remained well below the suggested diameter size limit of <100 μm which should enable incorporation into foods without affecting sensory attributes (Wang et al., 2015).

Encapsulation yields (EY) of 90-92% for incorporation of the bacterial payload into the TGase cross-linked G-MD microspheres were similar to EYs obtained for gelatin microspheres (Annan et al., 2008; Saravanan et al., 2011).

Images obtained through phase contrast microscopy and CLSM revealed that the microspheres contained a maltodextrin core surrounded by an outer gelatin layer with a thickness of about 16-20 μm (Figure 5.1). A similar structure was observed for genipin cross-linked G-MD microspheres made with the same proportion of gelatin to maltodextrin (10%/3%) (Borza et al., 2010) and could be explained by the phase separation between the two biopolymers (Lorén & Hermansson, 2000). The entrapped bacteria were in both studies mainly located in the interphase between the two phase separated biopolymers with some bacteria located in the gelatin outer core.

5.5.2 Comparison of survival of free and encapsulated cells in simulated gastric juice

An important purpose of microencapsulation is to increase the survival of probiotic lactic acid bacteria during exposure to the adverse gastrointestinal conditions after consumption, including a 2-3 h stay in the stomach in its low pH (1.5 to 3) environment (Liong and Shah, 2005). In the present study, we show that encapsulation in a mixed biopolymer matrix made with food grade gelatin, maltodextrin and TGase increased survival of the encapsulated cells in comparison to free cells. This increase in survival may be due to the buffering effect of gelatin, the location of the entrapped cells away from the surface of the phase separated microspheres and the structural stabilization afforded by the action of the TGase enzyme.

Encapsulation of three strains *Lactobacillus* spp. (3C2-10, 21C2-10, 21C2-12) in the TGase cross-linked microspheres made with gelatin A 300 and maltodextrin significantly ($p<0.05$) improved the survival by 4 orders of magnitude over that of free cells during exposure to SGJ (Figure 5.2c). This result was comparable to the protective

effect observed for genipin cross-linked G-MD encapsulated bifidobacteria (Borza et al., 2010). Importantly in that study, we also observed that the mixed biopolymer G-MD microspheres afforded more protection to the entrapped cells than gelatin-only microspheres. This could be due to the prebiotic effect of maltodextrin and the location of the bacteria towards the centre of the G-MD microsphere as opposed to towards the surface of the gelatin-only microspheres, where the exposure to the acid would be more pronounced.

Control of the enzymatic degradation of gelatin microspheres that could lead to premature release of the microencapsulated cells was in the present study achieved by augmenting the stability of physical (triple helices) cross-linkages (Meng and Cloutier, 2014) with the TGase introduced covalent N ε-(γ-glutamyl) lysine amide bonds to cross-link gelatin strands (Yung et al., 2007). Use of gelatin of increasing bloom strength (i.e., larger gelatin strands) improved the stability of TGase cross-linked G-MD microspheres during exposure to acidification and pepsin attack in simulated human gastric juice (Figure 5.4). The structural integrity of the microspheres during SGJ exposure correlated with survival of the entrapped bacteria as was also observed in a study where gelatin-only microspheres were protected from pepsin degradation in SGJ by an outer alginate layer (Annan et al., 2008). Guérin et al. (2003) reported that the buffering effect of whey proteins contributed to higher survival rates for LAB encapsulated in a mixed gel of alginate, pectin and whey proteins in comparison to free cells when exposed to SGJ at pH 2.5. Similarly, encapsulation in another protein-based microsphere (avg. diameter 22 μm) made with 0.1% alginate and 10% chickpea protein isolate was recently reported to protect *Bifidobacterium adolescentis* (ATCC 15703) during exposure to SGJ (Wang et al.,

2015), indicating the general promise of protein based microspheres.

The decrease in the viable population by 3.8-4.0 log units for free *Lactobacillus* spp. cells was similar to findings where reductions of about 3 log(CFU/ml) for *Lactobacillus* and *Bifidobacterium* strains exposed to SGJ (pH 2.0) for 2 to 3 h were observed (Charteris et al., 1998; Hansen et al., 2002). It should be noted, however, that *in vitro* studies with SGJ tend to overestimate viability losses that would occur *in vivo*, as it is known that the presence of food components may temporarily elevate the gastric pH (Mainville, Arcand & Farnsworth, 2005).

5.5.3 Survival of free and encapsulated cells during sequential incubation in simulated gastro-intestinal conditions

While exposure to bile in the intestinal tract can be highly toxic for microorganisms (Hansen et al., 2002; Ruiz et al., 2013; Mandal et al., 2014), many probiotics have evolved mechanisms such as bile salt hydrolases to neutralize the effect (Begley, Hill, & Gahan, 2006). In this study, almost all of the viability loss happened during exposure to SGJ for 1 h with only minor changes occurring during exposure to SIJ for 3 h (Figure 5.3). A study of *B. adolescentis* encapsulated in matrices consisting of different plant proteins (chickpea, soy, faba bean, and lentil) and alginate (0.1% w/w) similarly showed limited declines in cell viability during exposure to SIJ contrasting the 1 log loss observed for free cells (Wang et al., 2014). Pavunc et al. (2011) observed a 10% decrease in survival of entrapped *Lactobacillus helveticus* M92 after exposure to SIJ for 4 h as compared to a 15% reduction of free cells.

FAO has recommended that the viability of probiotics should at a minimum be 10^7 CFU/g of product at the time consumption (Morelli and Capurso, 2012). Encapsulation

of probiotics in protective microspheres made from food-grade ingredients, e.g., TGase cross-linked G-MD, may represent an alternative strategy for the delivery of live viable and efficacious lactic acid bacteria.

5.5.4 Viability of *Lactobacillus* strains in microspheres during refrigerated storage time

Microencapsulation maintained the viable numbers of three tested *Lactobacillus* strains during refrigerated storage for 40-57 days. The mixed biopolymer matrix made with gelatin and maltodextrin supported survival of the encapsulated cells in comparison to free cells. This improved survival during refrigerated storage might be due to carbon and nitrogen source from maltodextrin and gelatin. Capela et al., (2006) reported that the carrageenan mixed with maltodextrin improved survival of *L. rhamnosus* GGB103 during refrigerated storage. Yeo and Lion (2010) also found that the presence of soy milk supplemented with maltodextrin and pectin led to high viable number of *L. acidophilus, L. casei* and *Bifidobacterium longum* over 7 log units. This survival of bacterial cells, probably due to the ability of their bacterial strains produce the enzyme and hydrolyses maltodextrin to glucose for carbon source of bacterial growth. Moreover, the TGase cross-linked biopolymer matrix formation between gelatin and maltodectrin could protect the bacterial cells by forming the gel and reduce the loss of water activity leading to improved stability of the entrapped cells during refrigerated storage (Capela et al., 2006; Hebrard et al., 2010; Yeo and Liong, 2010). In this study, gelatin-maltodextrin microspheres were shown to enhance the viability of the entrapped bacteria over 40 day refrigerated storage.

5.6 Conclusions

Encapsulation in translucent G-MD microspheres crosslinked with TGase enhanced the survival of three *Lactobacillus* spp. strains (3C2-10, 21C2-10 and 21C2-12) during exposure to simulated upper gastrointestinal tract conditions, indicating the protective effect of the encapsulation matrix. The entrapped *Lactobacillus* spp. were released from the microspheres in the simulated intestinal juice to become available to exert their beneficial effects. The stability of viable entrapped *Lactobacillus* spp. was shown during 40 day at refrigerated storage. These finding demonstrated the TGase cross-linked G-MD microspheres harbor promise for use in functional foods and delivery of viable probiotics.

5.7 References

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CHAPTER VI

SUMMARY

Cholesterol-lowering probiotic bacteria were isolated from cassava pulp during the fermentation (0-28 days). Thirty eight isolates that showed BSH activities and tolerance to bile salt at concentration 0.15% and 0.50%, and pH 2 to 9 on MRS medium were selected for further studies. Live but not dead cells of the three selected strains (3C2-10, 21C2-10 and 21C2-12) could decrease cholesterol concentration in culture broth by 18-24 µg/ml. All three selected strains (3C2-10, 21C2-10 and 21C2-12) showed the strength of cell adhesion based on *in vitro* cell hydrophobicity and Caco2 cell lines, and could metabolize prebiotic such as fructooligosaccharides (FOS), lactulose and inulin. The strains were identified as *Lactobacillus plantarum*, *Lactobaillus acidophilus* and *Lactobacillus fermentum*, respectively.

Cassava pulp, brown rice and rice bran extracts were not detrimental to the growth of three selected strains (3C2-10, 21C2-10 and 21C2-12) and increased the survival of bacteria expored to simulated gastrointestinal conditions at pH 2 for 1h. In addition, the entrapment of the three *Lactobacillus* strains onto transglutaminase (TGase) cross-linked gelatin-maltodextrin (G-MD) microspheres sucessfully protected the probiotic lactic acid bacteria during exposure to simulated upper gastro-intestinal tract conditions. Microspheres made with gelatin A300 bloom and TGase concentration of 10 U/g showed the highest stability against pepsin-induced degradation of the microspheres in simulated gastric juice (pH 2.0, 2 h, 37 ^{o}C) and intestinal

juices (pH 7.4, 4h, 37^{o}C). The potentially cholesterol-lowering probiotic bacteria exhibited increased survive in simulated gasstrointestinal tract when encapsulated in transglutaminase (TGase) cross-linked.gelatin-maltodextrin (G-MD) microspheres. This microencapsulation process shows great promise for applications in food industry.

APPENDIX

Table 1 Hemolytic activity.

Strain	Hemolytic activity
3C2-10	-
21C2-10	-
21C2-12	-
0C4-4	-
1C4-3	-
1C4-11	-
L.plantarum strain 1465	-
L.plantarum strain 1463	-

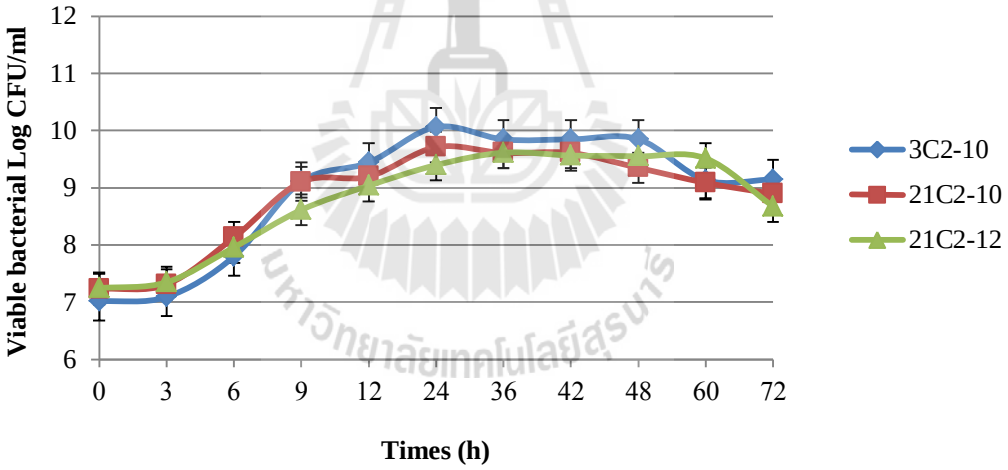

Figure 1 Growth of three isolated strains (3C2-10, 21C2-10 and 21C2-12) on MRS broth at 37° C for 72 hour.

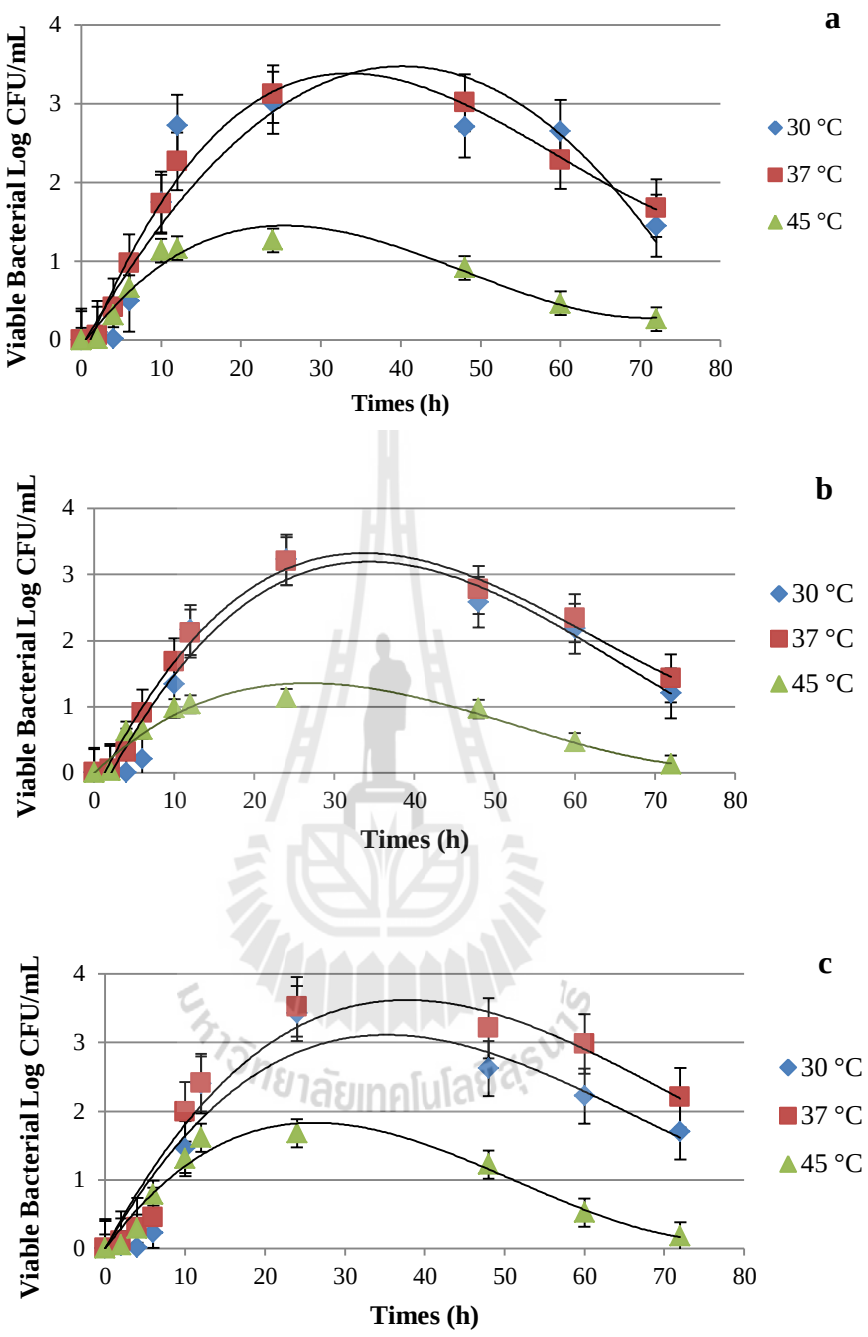

Figure 2 Growth of three isolated strains (3C2-10, 21C2-10 and 21C2-12) on MRS broth at 30° C, 37° C and 45° C, a) 3C2-10, b) 21C2-10 and c) 21C2-12.

CURRICULUM VITAE

Siriwan Nawong was born in Udonthani, Thailand. She attended Suranaree University of Technology, Thailand and received her Bachelor's degree in food technology in 2001. In 2001, she worked at Royal Home Food Products Co., Ltd. as a production supervisor. In 2002, she worked as research assistant in factors affecting histamine formation in fish sauce fermentation project under the grant No. SUT3-305-42-29-02. In 2003, she attended Suranaree University of Technology, Thailand and she was granted a scholarship by the National Center for Genetic Engineering and Biotechnology (BIOTEC) under the grant No. BT-B-06-FG-19-4603. In 2006, she received her Master's degree in food technology. In 2007-2014, she worked at Innovation and Technology Assistance Program (ITAP-SUT Network) as an industrial technology advisors. In 2010, she attended Suranaree University of Technology, Thailand and she was granted a scholarship by Research and Development Institute, Suranaree University of Technology, and National Research Council of Thailand who financially supported this research

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