ANTI-CANCER PROPERTIES OF *LACTOBACILLUS* SP. ISOLATED FROM CASSAVA PULP

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คุณสมบัติการยับยั้งมะเร็งของแลคโตบาซิลลัสซับสปีชีร์ซึ่งแยกได้จากกากมันสำปะหลัง

นางสาวสดุดี เส็งแสงทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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นางสาวสดุดี เส็งแสงทอง : คุณสมบัติการยับยั้งมะเร็งของแลคโตบาซิลลัสซับสปีชีร์ซึ่งแยกได้จากกากมันสำปะหลัง (ANTI- CANCER PROPERTIES OF *LACTOBACILLUS* SP. ISOLATED FROM CASSAVA PULP) อาจารย์ที่ปรึกษา :
ผู้ช่วยศาสตราจารย์ ดร. รัชฎาพร อุ่นศิวิไลย์, 133 หน้า.

วัตถุประสงค์ของการศึกษานี้ เพื่อศึกษาคุณสมบัติการยึดเกาะผนังลำไส้ใหญ่และการยับยั้งมะเร็งลำไส้ใหญ่ Caco-2 ของ *Lactobacillus* sp. 21C2-10 ซึ่งแยกได้จากกากมันสำปะหลัง และศึกษาผลของการห่อหุ้ม *Lactobacillus* sp. 21C2-10 ต่อคุณสมบัติทางเคมีกายภาพ จุลินทรีย์ ประสาทสัมผัสและความคงตัวต่อกระบวนการย่อยของไอศกรีม จากผลการศึกษาพบว่า *Lactobacillus* sp. 21C2-10 แสดงความสามารถในการเกาะติดผนังลำไส้ใหญ่ได้ดี อยู่ที่ 14.44±0.58% ยิ่งไปกว่านั้นสารเมทาบอไลท์ที่ *Lactobacillus* sp. 21C2-10 สร้างขึ้นเป็นพิษต่อเซลล์ไลน์ Caco-2 จากการทดสอบด้วยวิธี MTT assay โดยพบว่าความเป็นพิษต่อเซลล์ไลน์ Caco-2 ที่ก่อให้เกิดการตายนั้น เป็นผลมาจากการเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพอพโตซิส โดยยืนยันจากผลการทดสอบด้วยวิธี Tali imaged base cytrometry และการย้อมสีเซลล์ด้วยวิธี DAPI staining ยิ่งไปกว่านั้นจากการตรวจสอบการแสดงออกของยีนด้วยวิธี RT-PCR พบว่าการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิส เกิดจากการเพิ่มการแสดงออกของยีน *BAX, P53, Caspase-3, Caspase-8* และ *Caspase-9* และยับยั้งการแสดงออกของยีน *BCL-2* ของเซลล์ไลน์ Caco-2 แสดงให้เห็นว่า *Lactobacillus* sp. 21C2-10 มีคุณสมบัติในการยึดเกาะเซลล์มะเร็งลำไส้ใหญ่ และผลิตสารเมตา-โบไลท์ที่สามารถยับยั้งการเจริญของเซลล์มะเร็งลำไส้ใหญ่ผ่านกลไกการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิส จึงเหมาะสมเป็นอย่างยิ่งที่จะนำมาเป็นส่วนประกอบในผลิตภัณฑ์อาหารเพื่อสุขภาพ

จากผลการศึกษาคุณลักษณะของไอศกรีมที่ประกอบด้วย *Lactobacillus* sp. 21C2-10 ในรูปเซลล์อิสระ และเซลล์ที่ได้รับการห่อหุ้มระหว่างการเก็บรักษาด้วยการแช่แข็งที่อุณหภูมิ -20 องศาเซลเซียส เป็นเวลา 180 วัน โดยทำการห่อหุ้ม *Lactobacillus* sp. 21C-10 ด้วยเทคนิคอิมัลชันใช้มอลโตเดกซ์ตรินและเจลาตินเป็นวัสดุที่ใช้ในการห่อหุ้มเซลล์ อัตราการรอดชีวิต (%) ของ *Lactobacillus* sp. 21C2-10 หลังผ่านระบบทางเดินอาหารจำลองของไอศกรีมที่ประกอบด้วย *Lactobacillus* sp. 21C2-10 ในรูปเซลล์อิสระ และเซลล์ที่ได้รับการห่อหุ้มทำการตรวจสอบในวันที่ 180 ของการเก็บรักษา การประเมินทางประสาทสัมผัสของไอศกรีมทำการตรวจสอบวันที่ 1 และ 180 ของการเก็บรักษา จากผลการศึกษาพบว่าไอศกรีมที่ประกอบด้วยเซลล์ที่ได้รับการห่อหุ้ม แสดงอัตราการรอดชีวิต (%) และค่าพีเอชสูงกว่าไอศกรีมที่ประกอบด้วยเซลล์อิสระอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) หลังจากเก็บรักษาเป็นเวลา 180 วัน และไอศกรีมที่ประกอบด้วยเซลล์ที่ได้รับการห่อหุ้มมีปริมาณกรดที่ไทเทรตได้ (%) ต่ำกว่าไอศกรีมที่ประกอบด้วยเซลล์อิสระอย่างมีนัยสำคัญทาง

สถิติ ($p<0.05$) หลังจากเก็บรักษาเป็นเวลา 180 วัน ยิ่งไปกว่านั้นเซลล์ที่ได้รับการห่อหุ้มไม่มีผลต่อคุณสมบัติทางประสาทสัมผัสของไอศกรีมอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) และหลังจากผ่านสภาวะเลียนแบบกระเพาะอาหารเป็นเวลา 1 ชั่วโมง และลำไส้เล็ก เป็นเวลา 4 ชั่วโมง พบว่าไอศกรีมที่ประกอบด้วยเซลล์ที่ได้รับการห่อหุ้ม มีอัตราการรอดชีวิต (%) ของ *Lactobacillus* sp. 21C2-10 สูงกว่าไอศกรีมที่ประกอบด้วยเซลล์อิสระอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) จากผลการศึกษาดังกล่าวแสดงให้เห็นว่า *Lactobacillus* sp. 21C2-10 มีคุณสมบัติในการยึดเกาะเซลล์มะเร็งลำไส้ใหญ่ สามารถยับยั้งการเจริญของเซลล์มะเร็งลำไส้ใหญ่ผ่านกลไกการเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพอพโตซิส ยิ่งไปกว่านั้นเมื่อทำการห่อหุ้ม *Lactobacillus* sp. 21C2-10 สามารถปกป้องเซลล์โพรไบโอติกในผลิตภัณฑ์ไอศกรีม ระหว่างการเก็บรักษาด้วยการแช่แข็ง, หลังผ่านระบบทางเดินอาหารจำลอง ส่งผลให้ *Lactobacillus* sp. 21C2-10 มีอัตรารอดชีวิตในปริมาณสูงที่สามารถเพิ่มโอกาสในการแสดงคุณสมบัติการยึดเกาะ และการเหนี่ยวนำให้เกิดการยับยั้งเซลล์มะเร็งลำไส้ใหญ่ได้เพิ่มขึ้น

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา สุดตี เสี่ยงแสงทอง

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SADUDEE SENGSAENGTHONG : ANTI-CANCER PROPERTIES OF *LACTOBACILLUS* SP. ISOLATED FROM CASSAVA PULP.
THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph. D, 138 PP.

PROBIOTIC/CASSAVA PULP/ANTI-CANCER PROPERTIES

The objectives of this study were to investigate the cell adhesion and anti-cancer properties of *Lactobacillus* sp. 21C2-10 isolated from cassava pulp on Caco-2 cells. In addition, the effects of microencapsulation of *Lactobacillus* sp. 21C2-10 on the physicochemical, microbial, sensory and digestive stability properties of ice cream were studied. The results showed that *Lactobacillus* sp. 21C2-10 strengthened adherence to Caco-2 cells of 14.44±0.58%. Moreover, the secreted metabolites from *Lactobacillus* sp. 21C2-10 (SML) toxic to Caco-2 cells were analyzed through an MTT assay. The results revealed that cytotoxicity caused Caco-2 cells to die as a result of the induction of apoptosis which was confirmed by the Tali image based on the cytometry and DAPI staining tests. Moreover, the results of gene expression from RT-PCR assay showed significant effects on apoptosis by modulating an increased expression of *BAX, P53, Caspase-3, Caspase-8* and *Caspase-9,* and by inhibiting the expression of *BCL-2* on Caco-2 cell lines. There results confirmed that *Lactobacillus* sp. 21C2-10 had strengthened cell adhesion properties and that SML were able to show anti-cancer properties through the induction of the apoptosis pathway, indicating that this is an appropriate ingredient to use for functional food products.

The characteristics of ice cream containing free and microencapsulated *Lactobacillus* sp. 21C2-10 during frozen storage (-20^{0}C) was evaluated for 180 days. *Lactobacillus* sp. 21C2-10 was microencapsulated by an emulsion technique using maltodextrin and gelatin as wall materials. The survival rate (%) of *Lactobacillus* sp. 21C2-10 after exposure to the simulated gastro-intestinal condition of ice cream containing microencapsulated cells and free cells were evaluated after 180 days of frozen storage. Sensory evaluation of the ice cream was conducted after 1 day and 180 days of frozen storage. Ice cream containing microencapsulated cells showed a significantly ($p<0.05$) higher survival rate, lower acidity, and higher pH value compared to ice cream containing free cells after being stored for 180 days. The addition of microencapsulated cells had no significant ($p>0.05$) effect on the sensory properties of the ice cream. After exposure to simulated gastro-intestinal juices for 5 hours, the ice cream containing microencapsulated cells showed a significantly ($p<0.05$) higher survival rate compared to the ice cream containing free cells. These results indicate that *Lactobacillus* sp. 21C2-10 shows adhesion properties and an increase in anti-cancer properties as a result of apoptosis induction. Moreover, microencapsulation of *Lactobacillus* sp. 21C2-10 protected the microorganisms during frozen storage and after passage through simulated gastro-intestinal conditions delivered a high number of *Lactobacillus* sp.21C2-10 there was an increase in opportunities for adhesion properties and anti-cancer properties via the induction of the apoptosis pathway of the Caco-2 cells.

School of Food Technology Student's Signature Sadudee Sengsaengthong

Academic Year 2017 Advisor's Signature Ratchadaporn. O.

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LIST OF ABBREVIATIONS

%	=	Percentage
sp.	=	Species
log	=	logarithm
CFU	=	Colony forming unit
(μ, m, k) g	=	(milli, micro, kilo) Gram
(μ, m, μ) mL	=	(milli, micro) Liter
(μ, n) m	=	(micro, nano) Meter
^{o}C	=	Degree Celsius
t	=	ton
ha	=	hectare
ppm	=	part per million
LAB	=	lactic acid bacteria
i.e.	=	id est
min	=	Minute
SCFAs	=	Short-chain fatty acids
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	=	Phosphate buffered saline
W/O	=	Water in oil
O/W	=	Oil in water
W/O/W	=	multiple water-in-oil-in-water

LIST OF ABBREVIATIONS (Continued)

v/v	=	volume by volume
cat.no	=	catalog number
SML	=	Secreted metabolites from Lactobacillus sp. 21C2-10
(RT)-PCR	=	Reverse transcription polymerase chain reaction
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
cDNA	=	complementary DNA
qPCR	=	quantitative polymerase chain reaction
mRNA	=	Messenger RNA
w/v	=	weight by volume
rpm	=	Revolution per minute
kV	=	kilovolt
mM	=	(m, μ) M = (milli, micro) Molarity
secs	=	seconds
EC	=	Escherichia coli
MRS agar	=	De Man, Rogosa and Sharpe agar
PDA	=	potato dextrose agar
SPSS	=	Statistic Package for Social Sciences
SD	=	Standard deviation
Mmol	=	(milli, micro) Mole
PI	=	Propidium iodide
SEM	=	Scanning electron microscope

LIST OF ABBREVIATIONS (Continued)

ATP	=	Adenosine triphosphate
GC	=	Gas chromatography
mg/mL	=	Milligram per milliliter
EDTA	=	Ethylenediamine-tetraacetic acid
et al.	=	et alia (and others)

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Rational and background

In recent times, the demand for functional food has increased as consumers have become increasingly worried about their health (Mohammadi et al., 2011). Functional foods can be defined as food products that have the potential to promote beneficial effects for human health (Sousa et al., 2012). The market size for functional food products has rapidly expanded, with probiotic food products now taking a 30% share (Stanton et al., 2005).

Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO and WHO, 2002). The generality of probiotics are classified in the genera *Bifidobacterium* and *Lactobacillus* (Ouwehand, 2002). Some probiotics have been shown to be beneficial through the competitive exclusion of pathogens by adhesion to human gut mucosa, immune modulation and anti-inflammatory potential, induction of apoptosis pathways, anticarcinogenic activity (Shah, 2007), and other benefits (Burgain et al., 2011). Recently, *Lactobacillus* sp. has been used in probiotic food products, especially dairy products, such as ice cream, cheese and yogurt to provide beneficial effects (Burgain et al., 2011). As a guide for positive health, the International Dairy Federation has advised that probiotics are healty and should be present in a minimal quantity of 7 log CFU g-1 or mL-1 (Ouwehand and Salminen, 1998). However, the probiotics must be

stable during production, storage period and also throughout the gastrointestinal condition (Cruz et al., 2010).

Ice cream could be an alternate food vehicle for carrying probiotic bacteria to customers (Kailasapathy and Sultana, 2003). Ice cream has a neutral value of pH, and this offers the probability for protection of probiotics during storage periods (Christiansen et al., 1996). However, some authors have shown that the loss of probiotics due to the effect of freezing injury (Kailasapathy and Sultana, 2003). Therefore, this protection from food vehicles may be not enough for the protection of viable probiotic bacteria until target destination is reached. Thus, technologies have been discovered for the protection of viable probiotic bacteria using microencapsulation.

Microencapsulation helps to separate the probiotic bacteria from a harsh environment. Encapsulation is a technology that enhances the protection of probiotics from damage during processing, storage and, ultimately, transit through the digestive system (Ding and Shah, 2007). Sheu et al. (1993) reported that microencapsulation has often been recommended to increase viable probiotic cells during the freezing process and frozen storage. Some studies demonstrated that microencapsulated probiotic bacteria increasing survived in frozen milk (Kebary et al., 1998). Shah (2000) suggested that encapsulation improved the amount of viable probiotic bacteria in frozen yogurt and may increase the frozen dairy products's shelf-life. Consequently, microencapsulation technology can be used to prevent viability loss during the freezing step and during storage periods. The probiotic bacteria are loaded into capsules consisting of one or many various kinds of wall materials such as alginate, starch, soy protein and gelatin using methods such as emulsification and

extrusion (Dong et al., 2013). Also, the small size of microcapsules (<100 μm) prevents a negative affect on the sensory characteristics of the foods. Nawong et al. (2016) stated that encapsulation in maltrodextrin and gelatin which has been cross-linked by transglutaminase below 100 μm in size increased the survival of *Lactobacillus* sp. 21C2-10 during exposure to simulated gastrointestinal juices. Their results indicate that microcapsules are appropriate for use in functional foods in order to transport viable probiotic bacteria.

1.2 Research objectives

1.2.1 To investigate the cell adhesion properties of *Lactobacillus* sp. 21C2-10 on human colon cancer cell lines (Caco-2).

1.2.2 To investigate the cytotoxic effects of the secreted metabolites from *Lactobacillus* sp. 21C2-10 on Caco-2 cell lines.

1.2.3 To observe the effects of secreted metabolites from *Lactobacillus* sp. 21C2-10 on apoptosis induction of Caco-2 cell lines.

1.2.4 To increase the survival of potential probiotic *Lactobacillus* sp. 21C2-10 in simulated gastrointestinal conditions using emulsion as the microencapsulation technique.

1.2.5 To evaluate the physicochemical, sensory, and microbial characteristics of probiotic ice cream containing *Lactobacillus* sp. 21C2-10 in free and microencapsulated forms during frozen storage at -20ºC for 180 days.

1.2.6 To investigate the survival of the *Lactobacillus* sp. 21C2-10 in free and microencapsulated forms in ice cream during exposure to simulated gastrointestinal tract conditions.

1.3 Research hypothesis

Lactobacillus sp. 21C2-10 showed cell adhesion properties on Caco-2 and the secreted metabolites from *Lactobacillus* sp. 21C2-10 toxic to Caco-2 have anti-cancer properties via induction of the apoptosis pathway on the human Caco-2 cancer cell lines. In addition, the microencapsulated *Lactobacillus* sp.21C2-10 increases viable probiotics when there is a passage through simulated upper gastrointestinal tract conditions and during storage time of the ice cream product, which indicated a high number of *Lactobacillus*sp. 21C2-10 being active in the target organ.

1.4 Scope of this study

Lactobacillus sp. 21C2-10 were isolated from cassava pulp. The identification of *Lactobacillus* sp. 21C2-10 will be carried out (Nawong et al., 2014). The cell adhesion properties will be assessed (Duary et al., 2011). Preparation of the secreted metabolites from *Lactobacillus* sp. 21C2-10 following Duary et al. (2011) will be conducted for the study of cytotoxic effects and apoptosis induction properties on CaCo-2 colon cancer cell lines. *Lactobacillus* sp. 21C2-10 were microencapsulated using emulsion as the microencapsulation technique and gelatin and maltodextrin as wall materials (Nawong et al., 2016). The effects of the microencapsulation process on increasing the survival of *Lactobacillus* sp. 21C2-10 in probiotic ice cream will be investigated during storage time at -20ºC for 180 days and in simulated gastrointestinal conditions and. Also the effects of microencapsulated *Lactobacillus* sp. 21C2-10 on the physicochemical, microbiological and sensory characteristics of ice cream will be investigated.

CHAPTER II

LITERATURE REVIEW

2.1 Cassava

Cassava (*Manihot esculenta crantz*, Euphorbiaceae, Dicotyledons) is also known as manioc. The genus of *Manihot esculenta crantz* includes 98 species. *Manihot esculenta crantz* is the major planted group (Rogers and Appan, 1973; Nassar et al., 2008). It was first found in South America, then spread to Africa and afterwards it spread to tropical and subtropical areas in Asia (FAOSTAT, 2013). Cassava is the main tuber crop in more than 80 countries in the moist tropics and the sixth most popular crop after wheat, rice, maize, potato and barley (FAOSTAT, 2013). The yearly world yield of cassava is 129.02 million tons from 14.150 million ha from above 80 countries. Good export pricing of the European Economic Community (ECC) in 1980 helped promote Thailand's cassava production which resulted in Thailand becoming the largest exporter of cassava. The largest cassava crops in the world are produced in Thailand, Congo, Brazil, Nigeria and Indonesia (FAO, 2014). Cassava production in 2020 is expected to be approximately 291 million tons (Scott, Rosegrant, and Ringler, 2000). Montagnac et al. (2009) explained that the increase in cassava production comes from the demand for dried cassava and cassava food products from Asia and Africa respectively. Moreover, Okudoh et al. (2014) and Nguyen et al. (2007) stated that the growth of the cassava industry is due to the demand for cassava products in the African continent and the growth of the cassava

industry in Southeast and East Asia. From 2015 to 2016, cassava was cropped especially in Nakhon Ratchasima province in the north-east of Thailand (50%) (Thaitapiocastarchassociation, 2016).

50% of cassava is used to produce cassava starch, cassava chips, cassava pellets and cassava flour. Cassava utilization in Thailand is explained in Figures 2.1 and 2.2 which show the production process of cassava starch, starting with cassava roots being washed, then peeled, cut and mashed. The starch granules are extracted from cassava root by water. The starch solution is filtered and then dried in sunlight. The dried samples are then mashed. The cassava pulp is separated as solid waste (Veiga et al., 2016). Cassava pulp is remarkable for its value-added utilization. Recently, the cassava pulps are applied as animal feed, bioethanol, and soil conditioner (Anyanwu et al., 2015). Cassava pulp is a major waste product which is approximately 10-15% of cassava root (Sriroth et al., 2000). Cassava pulp includes 75% of moisture content. Its major components are (% dry basis) 55-56% of carbohydrates, 10-15% of fiber, 1.4-5% of protein, and 0.1-5% of fat (Kurdi and Hansawasdi, 2015; Shigaki, 2016). Moreover, cassava pulp also contains 155 ppm of Fe^{2+}, 40 ppm of Mn^{2+}, 1100 ppm of Mg^{2+}, 4 ppm of Cu^{2+}, and 21 ppm of Zn^{2+} per kg of dried cassava pulp (Kurdi and Hansawasdi, 2015). All data are shown in Table 2.1 (Thailandtopiocastarch, 2010).

Various studies used cassava pulp as a good substrate for microbial (bioprocessing), which is important for value-added utilization. Because cassava pulp is source of carbon, nitrogen and cofactor. In 2016, Nawong and et al. (2016) were able to isolate *Lactobacillus* spp. strains from cassava pulp in Nakhon Ratchasima province that have probiotic properties.

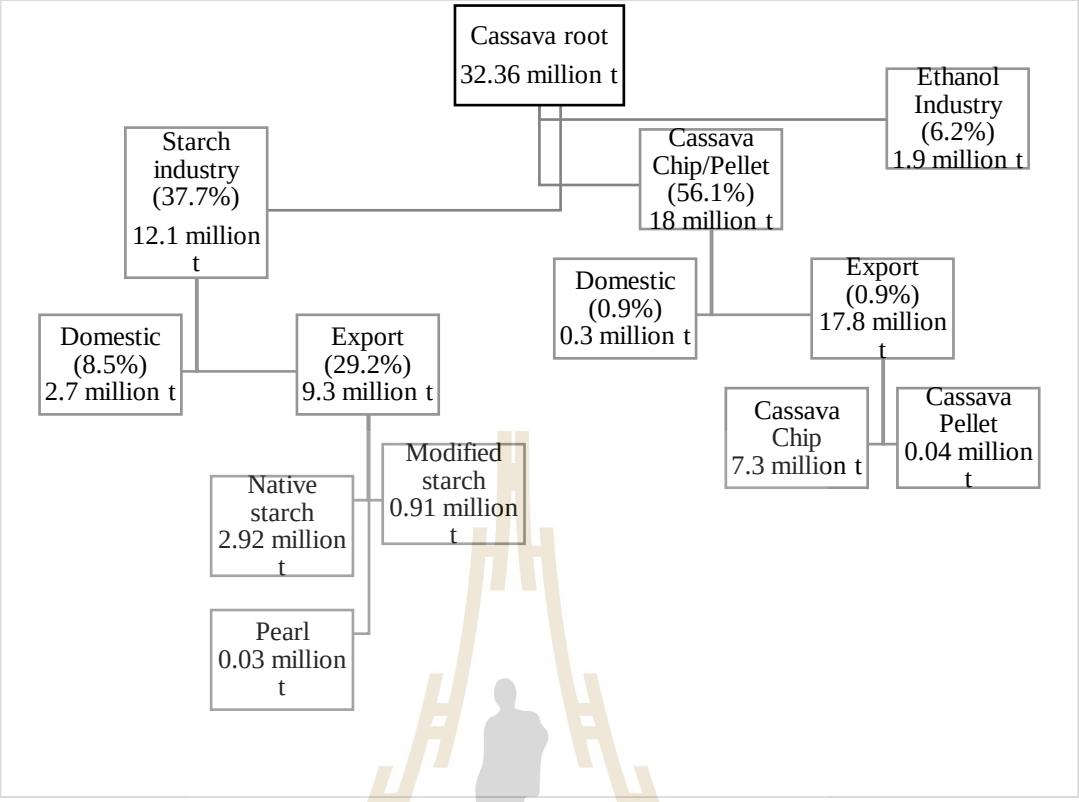

Figure 2.1 Cassava root utilization in Thailand in 2015.

(Office of Agricultural Economics, 2015).

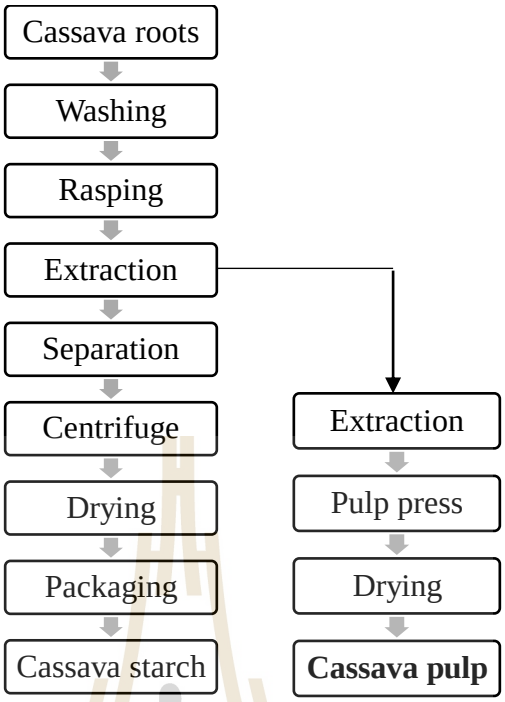

Figure 2.2 Flow diagram of cassava starch manufacturing process.

Table 2.1 Chemical composition of cassava pulp (% of dry weight).

Composition	**% of dry weight**
Moisture	9.25
Carbohydrate	48.72-0.07
Fiber	12.15-24.13
Protein	1.46-2.53
Fat	0.16-0.52
Starch	59.77
Minerals	**mg/kg**
Fe2+	155
Mn2+	4
Cu2+	4
Zn2+	21
Mg2+	1100

(Lohwongwatthana, 1982)

2.2 Probiotics

FAO/WHO defined probiotics as “Probiotics are live microorganisms which administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). Most probiotic bacteria have been shown to be lactic acid bacteria (LAB) group (FAO/WHO, 2002). Bourdichon et al. (2012) stated that LAB is a large group which is generally recognized as safe (GRAS) for food application. Lactic acid bacteria are gram-positive bacteria, non-spore forming, catalase-negative strain, and microaerophilic in strictly anaerobic conditions (Zhang et al., 2011; Ljungh and Wadstrom, 2006). The generality of LAB are *Lactobacillus, Bifidobacterium, Lactococcus* and others (Zhang et al., 2011). Further, characterization of probiotics must be investigated following probiotic potential and safety assessment of probiotics through in vitro tests, some of which are summarized in Table 2.2.

Moreover, *Lactobacillus* strains have been reported to provide health benefits such as antihypertensive effects, anti-carcinogenic properties, cancer prevention, anti-oxidative effects, treatment and prevention of gastrointestinal disorders, osteoporosis, obesity, atopic eczema, dental carries, ulcerative colitis, allergic symptoms, antibiotic-induced diarrhea and treatments of arthritis (Lee and Salminen, 2009). Several main health benefits of probiotics are summarized in Figure 2.4. However, *Lactobacillus* strains used as probiotics must be shown to be probiotic properties and both the phenotypic and genotypic should allow for safety criteria. *Lactobacillus* strains that show the following properties (FAO and WHO, 2002):

1. Strains are believed to originate in humans.
2. Non-pathogens.
3. No history of diseases caused by this strain.

4. They should not express antibiotic resistant genes.

Therefore, the addition of probiotics into food products could be used to modify foods to make them functional and to provide health benefits.

2.3 Acid and Bile tolerance properties

According to FAO/WHO (2002) "probiotic organisms used in food must be able to surviving passage through the gut; i.e., they must have the ability to resist gastric juices and exposure to bile". Probiotic bacteria are required to survive until transit to the target organs in the gastrointestinal tract (GI). Tolerance to acid and bile are believed to be major probiotic properties that should be viable and able to reach the target organs. Normally, foods enter the mouth and then pass through the gastrointestinal tract. Therefore, *Lactobacillus* spp. should be tolerant in the digestive system. Ruiz et al. (2013) reported that the stomach conditions are acidic and the pH is around 1.5-3.0 and the upper intestine condition is 0.05-2.00% of bile. Tolerance to acid and bile are important properties for probiotic selection while most researchers use in vitro tests for the study of the acid and bile tolerance of bacteria. In vitro tests use models of the gastrointestinal tract. These models can be used to obtain information regarding the potential of bacteria to survive in gastrointestinal conditions. Most researchers use test-tube assays to simulate the gastric and intestinal conditions (Gamboa and Leong, 2013). Samples can be taken studies from the model of the gastrointestinal tract to study the survival of bacteria. This information helps food producers to develop products that ensure appropriate numbers of viable probiotic bacteria reach the site of action (Farnworth, 2008). Thus it is useful to measure the probability of microorganisms being able to survive in many parts of the

gastrointestinal condition. It shows the tolerance of probiotic to bile and other acids. After mastication, the primary bacteria must survive in the stomach (low pH) for 60 min. Then it will pass through the intestinal tract (1.5-2.0% of bile acid) for 2.5-3.0 hours (Davis et al., 1986) and transit through the colon (Sahadeva et al., 2011). Therefore, probiotic bacteria for most uses can be simulated in vitro tests with GI conditions. Bacteria are counted by culture techniques which show the survival results of probiotics depending on the strain and species (Davis, 2014).

Many studies (Govender et al., 2014) suggest *Lactobacillus* sp. is resistant to when passing through the gastrointestinal tract. Survival of *Lactobacillus plantarum* NCIMB8826 after exposure to simulated gastric juices for 30 min shows a cell loss of only 1.92 Log CFU/ml (Michida et al., 2006). Similarly, Blaiotta et al., (2013) demonstrated *Lactobacillus paracasei* spp. showed more than 50% of survival after exposure to gastro-intestinal conditions. Ruiz et al. (2013) reported that tolerance to acid and bile promote the survival of *Lactobacillus* sp. when passing through the digestive system. Moreover, Nawong et al. (2016) suggest that *Lactobacillus* sp. isolated from cassava pulp shows acid and bile tolerance properties. The acid tolerance mechanism during microbial growth using multi subunit F0F1-ATPase activity. *Lactobacillus* sp. can survive in acidic conditions due to remaining cytoplasmic pH at close to neutral (Xia et al., 2011). The F0F1-ATPase shows a significant role in live cells, at low pH of cytoplasm. It is activated and decreases the pH of intracellular space by proton pump activity (Corcoran et al., 2005). The F0F1-ATPase is a major factor for acid tolerance mechanism of probiotic bacteria (Cotter and Hill, 2003). However, the activity of F0F1-ATPase is connected to the energy (ATP) (Liu et al., 2015). The bile acids (conjugated form) are released into the

duodenum at 500-700 ml/day (Ruiz et al., 2013). Bile acid can influence the viability of probiotic bacteria. Margolles et al. (2003) suggests bile acid in de-conjugated forms are more toxic to gram positive bacteria than gram negative bacteria (Margolles et al., 2003). Generally, microorganisms can not adapt to intestinal conditions because of the toxicity of bile. The mechanisms of probiotic bacteria are resistant to bile acid such as *Lactobacillus* spp. as a probiotic. Zhu et al. (2006) suggest that bile acid is toxic for probiotic bacteria and that many probiotic bacteria have bile salt hydrolase to reduce bile salt. However, Ruiz et al. (2013) report that acid and bile tolerance of *Lactobacillus sp.* depends on species and the different strains of microorganisms.

Therefore, acid and bile tolerance are important for stability and resistance of viable probiotic bacteria in bile and acid due to large amount of probiotics which pass through the gastro-intestinal tract and provide health benefits.

2.4 Adhesion property

The adhesion properties of probiotics to epithelial cells has been investigated which is most important for the selection of probiotic bacteria. Probiotics probably have a long-standing existence in the system (Nishiyama et al., 2016). The adherence of bacteria to epithelial cells probably protects probiotic bacteria from being rinsed off and capacitate transient colonization, competitive displacement of pathogens and modulation of immune system and increases the possibility of probiotic strains providing health benefits. Therefore, retarding the adhesion properties of pathogens probably results in microbial competition between probiotic bacteria and pathogens at similar receptors (Sánchez et al., 2008).

Table 2.2 Procedure for the isolation and characterization of novel strains with putative probiotic status.

Functional aspects	**Safety assessment**
1. Resistance to gastric conditions (acid and bile)	1. Antibiotic resistance
2. Adherence to mucus and/or human epithelial cells	2. Assessment of metabolic activities
3. Antimicrobial activity against potential pathogens	3. Production of toxic compounds
4. Inhibition/displacement of pathogen adhesion	4. Hemolytic potential
5. Modulation immune system	
6. Bile salt hydrolase activity	
7. Glycosidase activity	

(Lee et al., 2008)

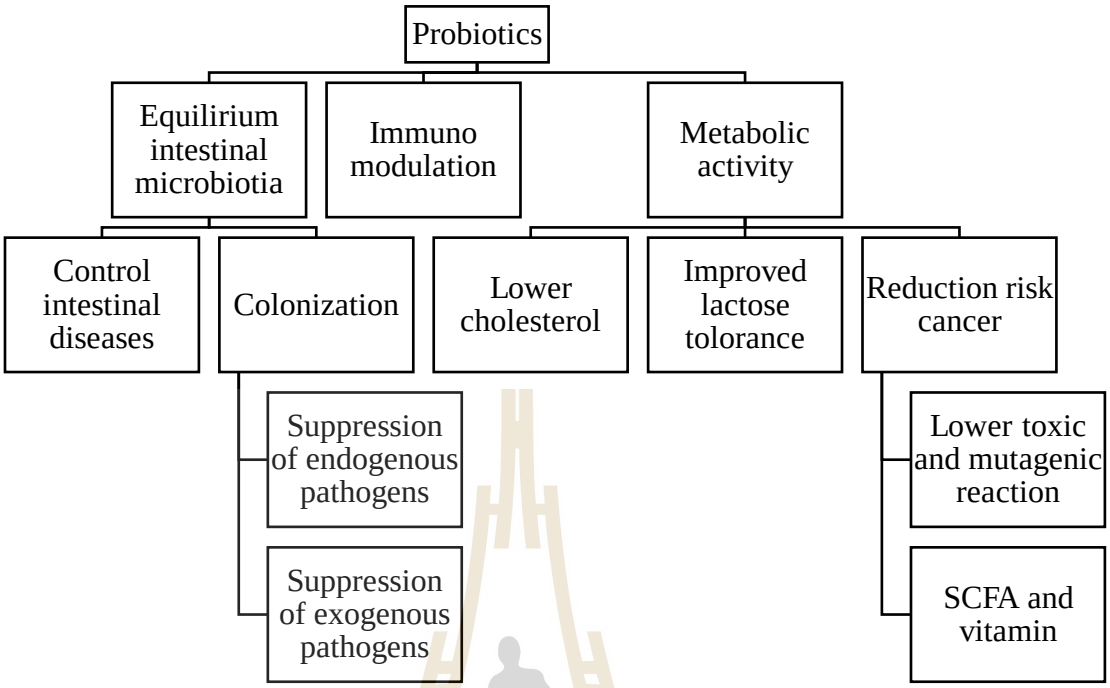

Figure 2.3 Beneficial effects of probiotics on human health. (Lee et al., 2008)

Many assays have been applied to the assessment of probiotic adhesion properties (Dimitrov et al., 2014). The most usual are the tests evaluating the adherence to epithelial cell of human culture cell lines (Table 2.4). An original assay for evaluating cell adhesion of probiotic cells is usually tested with Caco-2. *Lactobacillus* strains inhibit the adhesion to intestinal epithelial cells (HT-29) of pathogens such as *E.coli* which increases the expression of MUC3 gene (Mack et al. (2003)), which inhibits the adhesion of *E. coli* (Collado et al., 2007). Moreover, Roos and Jonsson (2002) reported that the major significant adhesins of probiotics are surface proteins of *Lactobacillus*, for example, the present of a peptidoglycan layer, polysaccharide, cell surface protein and others which also have adhesion properties (Barreiro et al., 2008). Furthermore, the physicochemicals of the cell surface of

bacteria cells include the glycoprotein effect which is highly hydrophobic and the hydrophilic group in the cell surfaces are connected with the polysaccharides of mucin or epithelial cells (Sánchez et al., 2008; Veléz et al., 2007). Moreover, the adhesion of probiotic cells is related to a variety of biochemical properties of probiotic bacteria and epithelial cells, including hydrophobicity, electrostatic interactions and cellular structures (Servin and Coconnier, 2003) and these adhesion properties of probiotics which can create biofilm and bio-surfactants (Cortes-Sanchez Ade et al., 2013).

In summary, the evaluation of the adhesion properties of probiotic bacteria compete with pathogens and provide increasing opportunities to demonstrate the health benefits for humans. They have most impact on selected probiotic bacteria.

Caco-2 human intestinal cell model

Human colorectal cancer cell lines (Caco-2) are cell cultures which originate from human colorectal adenocarcinoma that show certain characteristics both morphological and functional which are similar to ordinary small intestinal enterocytes (Sambruy, Ferruzza, Ranaldi, and De Angelis, 2001). The characteristics of Caco-2 cell lines are shown in Table 2.3. Pinto et al. (1983) reported that Caco-2 cell lines can be differentiated into an enterocyte when monolayers obtain cell confluent using general conditions. Differentiation during early stages in the Caco-2 cell lines expresses proteins such as colonocytes and enterocytes. Differentiation decreases the expression of colonocyte-specific genes and morphological alterations including biochemical characteristics of developed enterocytes whereas after about two weeks, the monolayer is featured by greatly polarized columnar epithelial cells with tight junctions and desmosomes with separate microvillar membrane from the basolateral membrane. Moreover, enzymes, for example, dipeptidylpeptidase IV,

lactase and sucrase-isomaltase are generally appear in the membrane of enterocytes brush border, however, they are not found in colonocytes. Most studies favour using Caco-2 cell lines for screening cytotoxicity and studies about anti-cancer properties in a colon cancer model with an in vitro test (Prakash et al., 2013).

Table 2.3 Properties of Caco-2 cell cultures.

Biological source	**Human colon (Caucasian colon adenocarcinoma)**
Growth mode	Adherent
Karyotype	Hypertetraploid, modal no. 96
Morphology	Epithelial
application(s)	cell culture | mammalian: suitable

(Failla and Chitchumronchokchai, 2005)

2.5 Cytotoxicity

Cytotoxicity assays are generally assessed for the evaluation of drugs and the cytotoxicity of substances, which are extensively used in vitro tests for toxicology studies. Mosmann (1983) has developed a quick colorimetric method which depends on the use of tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), which determines only viable cells and observations using a multiwell spectrophotometer (ELISA reader). The MTT assay evaluates the potentiality of viable cells to diversify a tetrazolium dye MTT (soluble) into a precipitated formazan (insoluble), which tetrazolium salt MTT receives electrons from oxidized substrates or appropriate enzymes such as NADH and NADPH (Berridge and Tan, 1993). This interaction changes the tetrazolium salts (yellow) into formazan crystals (blue) that

can be dissolved in an organic solvent (Figure 2.5). The formazan crystals are insurmountable to the membrane of cells and therefore it stores in complete cells. Consequently, cell viability can be detected by determining the concentration of formazan in optical density using an ELISA plate reader (570 nm). Recently, an MTT assay was evaluated in many cell lines (Mosmann, 1983). Due to the various strengths of this assay, it is regarded as important progress beyond conventional techniques. In reality, it is quick, easy-to-use, secure, multipurpose, quantitative, easy to duplicate and extensively used in cell viability and cytotoxicity tests. Consequently, this method is appropriate for preliminary works (Supino, 1995). Cytotoxicity in cell culture is typically expressed by cell viability (%). Various researchers have studied in vitro cytotoxicity using the MTT assay for evaluation of the anti-proliferative effects of probiotic bacteria on cancer cells (Grimoud et al., 2010; Ouwehand, 2011). The present research emphasizes the anti-proliferative properties of human colorectal colon cancer cell lines by probiotics (De Moreno De Leblanc and Perdigon, 2010). The metabolites secreted from *Lactobacillus acidophilus* have the most potent anti-proliferative effects or cytotoxicity effect on HT-29 cell lines (Nami et al., 2014). Similar research was reported by Haghshenas et al. (2014); Haghshenas et al. (2015); Saxami et al. (2016); Orlando et al. (2016) suggest that bacterial secreted metabolites from probiotic bacteria had anti-proliferative effects or cytotoxicity effects against human gastrointestinal cancer cells by induction of apoptosis.

Nowadays, studies about secreted metabolites from microorganisms favour testing cytotoxicity using an MTT assay for screen anticancer properties before the test with mechanism for cell deaths.

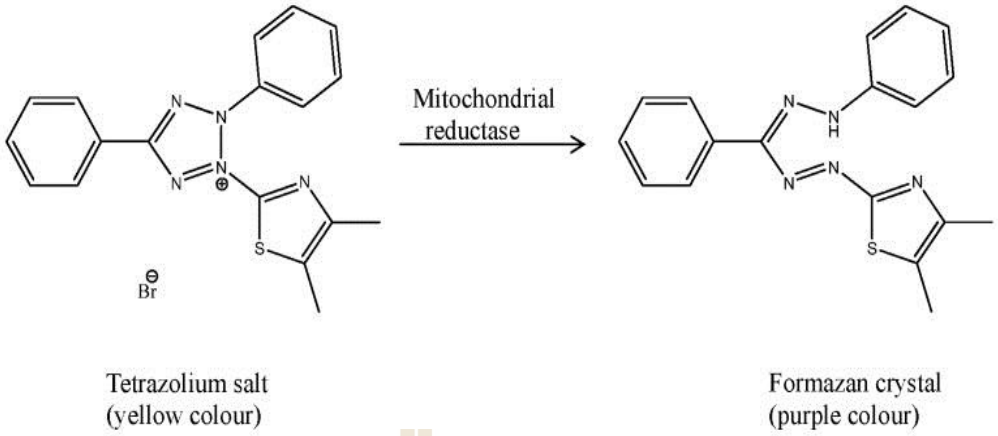

Figure 2.4 Chemical structure of yellow MTT and purple formazan products in living cells (Supino, 1995).

2.6 Anti-cancer properties

Colorectal cancers (CRC) are the third major form of the disease in men's lung and prostate cancers and in women's breast and lung cancers. CRC is a major form of cancer worldwide, recording more than 1 million cases and approximately half a million deaths yearly (Arnold et al., 2017). However, rates of CRC are predicted to increase the number of new case by more than 2.2 million in 2030 (worldwide) (Arnold et al., 2017), recommending that CRC still a main worldwide diseases load (Turati, 2017). The major factor in the occurrence of colorectal cancer (CRC) is diet factor at 35% (Peterson et al., 2014). Epidemiological studies show that there is a relationship between the appearance of colon cancers and the quality of foods (Mennigen et al., 2009). Various epidemiologic studies have shown the relationship between the nature of diets and the occurrence of CRC, with foods including fats, cholesterol and proteins from animals proving carcinogenic in trial studies (Lichtenstein, 1998).

Table 2.4 Adhesion of probiotics to gastrointestinal epithelial cell lines (Cac0-2).

Probiotics	**Results**
Lactobacillus GG and other dairy strains	*Lactobacillus* GG more adherent than other tested dairy strains
Human *Lactobacillus* isolates	Adhesion strain specific; dependent on age of the cell culture
Lactobacillus acidophilus LA1 and three other strains	Adhesion calcium independent
Commercial probiotic and dairy strains	Adhesion strain specific
Various *lactobacilli* and bifidobacteria	Adhesion does not affect cytokine production by epithelial cells
Eight *Bifidobacterium* strains	Caco-2 cells respond differentially to *E. coli* and *bifidobacterium*; response independent from adhesion
Thirteen *Bifidobacterium longum*	Adhesion correlated with autoaggregation properties

(Lee and Salminen, 2009; Barahuie, Hussein, Fakurazi, and Zainal, 2014).

Probiotics are able to provide for many health supporting effects, one of which is their anti-cancer properties. Their relation with the protection of cancer in animals and humans has been thoroughly researched. Previously, the effects of probiotics or food products containing LAB were often studied for anti-colon cancer properties

(Rafter, 2004). There is evidence demonstrating that dairy products containing *Lactobacillus* spp. possibly have a function in protecting the colon from cancer. However, these mechanisms are not yet fully understood and may be related to various factors. Howarth and Wang (2013) reported that the ability to reduce the risk of CRC by induction of apoptosis, stimulated the immune system, decreasing infection, controlling inflammation and binding with toxic compounds. Many studies have suggested metabolite products of *Lactobacillus casei* ATCC334 inhibit cancer proliferation in human colorectal cancer cell lines as Caco2, SKCO-1, and SW620 cells (Escamilla et al., 2012). In addition, the fermented supernatant from probiotic bacteria showed important anti-proliferation effects and apoptosis induction effects in HT29 cell lines (Markowiak and Slizewska, 2017). It is clear from this research that metabolites of probiotic bacteria decrease colon cancer proliferation. They may play a role in decreasing the risk of colon cancer from short chain fatty acids (SCFAs) by probiotic bacteria fermentation such as butyrate, acetate and propionate which produce probiotic bacteria and display mechanisms which possibly help the prevention and treatment of colon cancer. SCFAs are metabolized by the fermentation of substrates by probiotic bacteria and they lead to induction of apoptosis pathways of colorectal colon cancer cell lines (Zeng et al., 2014). SCFAs inhibit cancer proliferative effects via blocked histone deacetylase and increased apoptosis in cancer cells. Jan et al. (2002) showed that Propionibacterium is related to the induction of apoptosis pathways in colon cancer cell lines (HT29 and Caco2) via SCFA that are produced by fermentation. Lastly, secreted metabolites of probiotic bacteria provide anti-proliferative effects of SCFAs such as inhibiting gene expression of pro-inflammatory genes, and COX-2 down-regulation (Corrêa-Oliveira et al., 2016). It has

been suggested that secreted metabolites from probiotic bacteria could affect anti-cancer from many pathways especially apoptosis pathways.

2.7 Apoptosis property

2.7.1 Apoptosis pathway

Apoptosis pathway is a significant function in the controlling of cell proliferation and cell death (Elmore, 2007). However, colon cancer resistance to cell apoptosis and un-controlling of cell proliferation are the major characteristics. Therefore, the controlling of cell proliferation and induction of cell death can be used for cancer prevention and treatment methods (Gerl and Vaux, 2005). Furthermore, probiotics induce cell apoptosis which is of considerable interest since cancer cells would be disposed of and because apoptosis may result in no damage to the nearby cells and not cause any cell inflammation, which is dissimilar from other treatments using chemo and radio therapy. The apoptosis pathway can be classified into two pathways which are intrinsic and extrinsic as shown in Figure 2.6.

2.7.2 Intrinsic pathway

The intrinsic signaling pathway or mitochondrial pathway that begins apoptosis is activated with many stimulators consisting of DNA-damaging substances or stress of cytotoxic, cellular and mitochondria caused by DNA damage, heat shock and others (Hassan et al., 2014). The stimulator begins the intrinsic pathway by making intracellular signals that are related to external growth factors, viral infections, cytokines, toxins, radiation, free radicals and hormones that can lead to a failure of repression of the programmed cell deaths. Therefore, triggering apoptosis were occurred through release of cytochrome c. So that apoptosis pathway can take place.

The signals regulate group of BCL-2 family such as a group of anti-apoptotic proteins including BCL-2, BCL-XL and a group of pro-apoptotic proteins, including Bid, Bax, Bak, which have the effect of breaking the mitochondrial membrane and leaking of cytochrome c leaks into the cytosol. A relation of cytochrome-c, apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9 bluids an apoptosome, then activates caspase-9 and downstream of effector caspases consisting of Caspase-3, and Caspase-7, which provides apoptosis pathways. Therefore, Cory and Adams (2002) suggested controlling the mitochondrial apoptosis through groups of the BCL-2 proteins. Moreover, Smac/Diablo proteins inhibit apoptosis proteins (IAPs) (Apraiz, Boyano and Asumendi, 2011) for which the intrinsic pathway protein is shown in Table 2.5. Furthermore, Schuler and Green (2001) reported the P53 as tumor suppressor protein has a significant function in the control of the BCL-2 proteins groups for induction of apoptosis pathways. However, certain mechanisms have not yet been fully clarified.

2.7.3 Extrinsic pathway

The extrinsic pathways that begin apoptosis are related to the interaction of receptor-mediated signaling. Table 2.6 shows the most common extrinsic pathway proteins used for either protein. The molecules signaling is called ligands, which are released from other cells, then attack the transmembrane death receptors on the target organ for induction of apoptosis pathways. Ashkenazi and Dixit (1998) reported this pathway consists of cell surface receptors appertaining to the death receptors (TNF receptor, Fas/APO1/CD95) which trigger apoptosis with the binding of ligands. The groups of the receptor in an extracellular cytoplasmic domain are known as the "death domain". The death domain presents an important function in communicating the death signaling from the cell surface to the intracellular

(Ashkenazi et al., 1998). Rubio-Moscardo et al. (2005) reported the compatible death receptors included FasL/FasR, TNF-α/TNFR1 and others (Ashkenazi et al., 1998). The sequence that assigns the apoptosis extrinsic stage is excellently featured with the FasL/FasR models. This model of receptors groups and binds with a similar trimetric ligand, when binding of ligand provides adapter proteins in cytoplasmic are enrolled which show death domains binding with the receptors. The Fas ligand binding to Fas receptor effects the adapter protein FADD binding (Wajant, 2002). FADD is related to procaspase-8 through the dimerization of the death effector domain, then a death-inducing signaling complex or DISC is created which affects the procaspase-8 stimulation (Kischkel et al., 1995). Whenever caspase-8 is activated, the apoptosis performance step is triggered. The activated caspase-8 activates the downstream effectors caspase-3 and -7, leading to apoptosis pathways. Caspase-8 also activates the Bid protein, which translocates to mitochondria, activates BAK or BAX and results in cytochrome c releasing in the route efficient to be inhibited by BCL-2 for induction of apoptosis. An apoptosis pathway or programmed cell death can occur quickly and is a possible treatment for the prevention of many types of cancer, for example, apoptosis pathways can be used for anti-cancer on colon cancer.

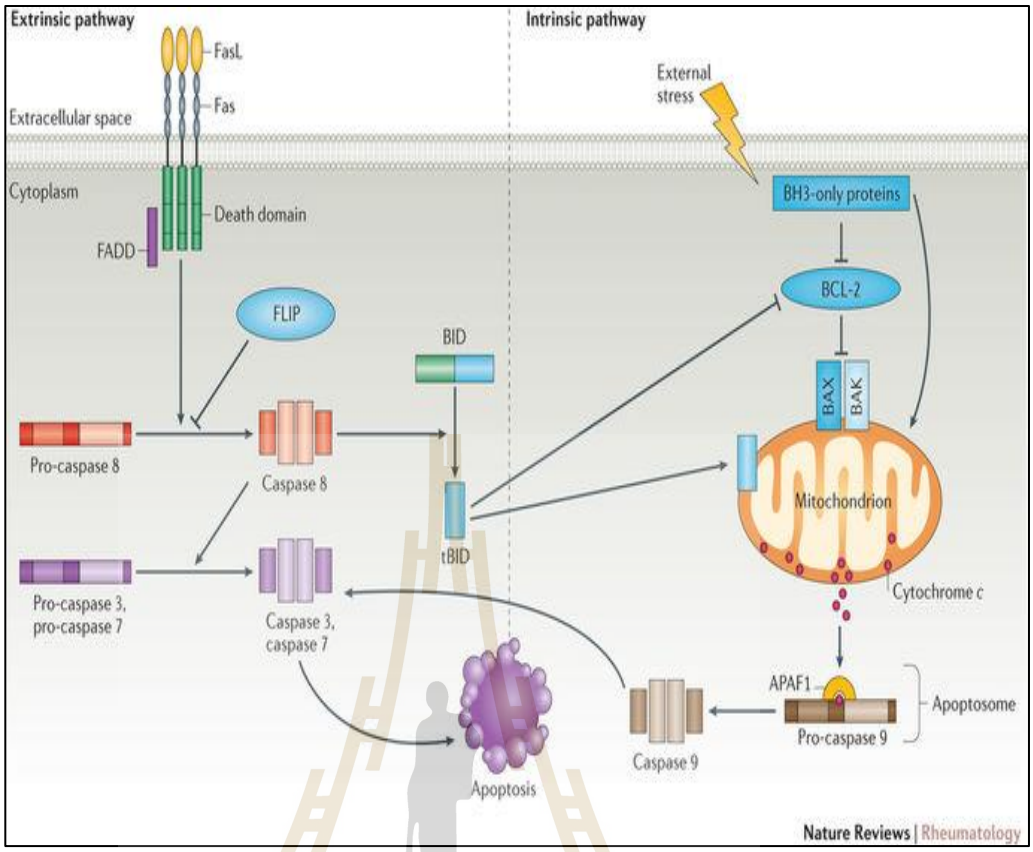

Figure 2.5 Intrinsic or mitochondria and extrinsic or death receptor signaling pathways. (Cuda et al., 2016)

Table 2.5 Intrinsic pathway proteins, abbreviations, and alternate nomenclature.

Abbreviation	Protein Name	Select Alternate Nomenclature
Smac/DIABLO	Second mitochondrial activator of caspases/direct IAP binding protein with low PI	None
IAP	Inhibitor of Apoptosis Proteins	XIAP, API3, ILP, HILP, HIAP2, cIAP1, API1, MIHB, NFR2-TRAF signaling complex protein
Apaf-1	Apoptotic protease activating factor	APAF1
Caspase-9	Cysteinyl aspartic acid-protease-9	ICE-LAP6, Mch6, Apaf-3
AIF	Apoptosis Inducing Factor	Programmed cell death protein 8, mitochondria
Bcl-2	B-cell lymphoma protein 2	Apoptosis regulator Bcl-2
Bcl-XL	BCL2 related protein, long isoform	BCL2L protein, long form of Bcl-x
BAX	BCL2 associated X protein	Apoptosis regulator BAX
BAK	BCL2 antagonist killer 1	BCL2L7, cell death inhibitor 1
BID	BH3 interacting domain death agonist	p22 BID

(Elmore, 2007)

Table 2.6 Extrinsic pathway proteins, abbreviations, and alternate nomenclature.

Abbreviation	Protein Name	Select Alternate Nomenclature
TNF-α	Tumor necrosis factor alpha	TNF ligand, TNFA, cachectin
TNFR1	Tumor necrosis factor receptor 1	TNF receptor, TNFRSF1A, p55 TNFR, CD120a
FasL	Fatty acid synthetase ligand	Fas ligand, TNFSF6, Apo1, apoptosis antigen ligand 1, CD95L, CD178, APT1LG1
FasR	Fatty acid synthetase receptor	Fas receptor, TNFRSF6, APT1, CD95
Apo3L	Apo3 ligand	TNFSF12, Apo3 ligand, TWEAK, DR3LG
DR3	Death receptor 3	TNFRSF12, Apo3, WSL-1, TRAMP, LARD, DDR3
Apo2L	Apo2 ligand	TNFSF10, TRAIL, TNF-related apoptosis inducing ligand
DR4	Death receptor 4	TNFRSF10A, TRAILR1, APO2
DR5	Death receptor 5	TNFRS10B, TRAIL-R2, TRICK2, KILLER, ZTNFR9

(Elmore, 2007)

Table 2.6 Extrinsic pathway proteins, abbreviations, and alternate nomenclature (continue).

Abbreviation	Protein Name	Select Alternate Nomenclature
FADD	Fas-associated death domain	MORT1
TRADD	TNF receptor-associated death domain	TNFRSF1A associated via death domain
RIP	Receptor-interacting protein	RIPK1
DED	Death effector domain	Apoptosis antagonizing transcription factor, CHE1
caspase-8	Cysteinyl aspartic acid-protease 8	FLICE, FADD-like Ice, Mach-1, Mch5
c-FLIP	FLICE-inhibitory protein	Casper, I-FLICE, FLAME-1, CASH, CLARP, MRIT

(Elmore, 2007)

2.7.4 Apoptosis induction by probiotics

The fermentation of substrates by probiotic bacteria produces short chain fatty acids (SCFA), which have an anti-proliferative effect through the induction of apoptosis pathways of colon human cancer (Uccello et al., 2012). The anti-proliferative effect in colon cancer cells by *Lactobacillus* sp. through induction of

apoptosis pathway (Chen et al., 2011; Nami et al., 2014; Thirabunyanon, Boonprasom, and Niamsup, 2009). In vitro tests, *Lactobacillus fermentum* RM28 isolated fermented milk were efficient in the inhibition of Caco-2 cell of 23% (Thirabunyanon, Boonprasom and Niamsup, 2009). Similarly, Sadeghi-Aliabadi et al. (2014) reported an anti-proliferative activity of supernatants of *Lactobacillus plantarum* A7 and *Lactobacillus rhamnosus* GG via apoptosis pathways. Baldwin et al. (2010) and Cousin et al. (2012) suggested that probiotic bacteria were efficient in increasing induction of apoptosis in many colon cancer cell lines resulting in probiotic bacteria controlling anticancer activity. *Lactobacillus* spp. induces cytotoxicity via induction of apoptosis on colon cancer cell lines. In addition, Wan et al. (2014) observed the possibility of inducing apoptosis of SW620 cell line by *Lactobacillus delbrueckii* was increased by *Caspase-3* gene expression and decreased by *BCL-2* gene expression. The supernatants obtained from fermentation of *Lactobacillus delbrueckii* decreased the cell proliferation of colon cancer cells (SW620 cell lines) through cell cycle arrest and induced apoptosis via the intrinsic pathway by decreasing the expression of the *BCL-2* gene. Kim et al. (2008) reported *Bifidobacterium adolescentis* SPM0212 inhibited the proliferation of HT-29, SW480, and Caco-2 via inhibited TNF-a production.

Moreover, Canani et al., (2011) reported SCFA as produced from probiotic bacteria could inhibit activity of the histone deacetylase enzyme that provides hyper acetylation to histones. The histone hyper acetylation effect in up-regulation of different genes connected to the cell cycle controlling, induction of apoptosis, and the development of cancer. Furthermore, SCFA affects the expression of Bcl2 family proteins, and triggers apoptosis through the up-regulation of *BAK* and the down

regulation of *BCL-XL* genes. There have been many studies on the effects of butyrate on CRC as it helps to regulate the balance between proliferation, differentiation, and apoptosis in cancer cells (Bishop, Xu and Marlow, 2017) It also stimulates the activity of proteins related to apoptosis including *BCL-2*, *BAK*, and *Caspase-3* and *Caspase-7* (Kumar et al., 2013). Furthermore, SCFA can also regulate extrinsic pathway in human colorectal cancer cells (Tang, Chen, Jiang, and Nie, 2011). *Propionibacterium freudenreichii* has been shown cause death as a result of cancer of the human colon via the short chain fatty acids (SCFA) in the media. Secreted metabolites from probiotic bacteeria as well as pure SCFA induces symbolic signals of apoptosis like activation of caspase-3and condensation of nuclear chromatin (Jan, Belzacq, Haouzi and Brenner, 2002).

As a result of these findings, probiotics are known to repress the CRC proliferation efficiently through the induction of apoptosis pathway by affecting different signaling pathways. However, in vitro assessments show the effects of SCFA from the fermentation by probiotic bacteria secretes metabolites to the external environment cause anti-cancer by the induction of apoptosis which are important mechanisms in the protection and treatment of cancer and can provide anti-cancer in many pathways especially induction of apoptosis pathways.

2.8. Encapsulation techniques

Encapsulation techniques are appropriate methods for delivering active compounds to the target organ. Encapsulation is defined as "a process to entrap an active compound within a stable, protective substance to produce encapsulates of varied size and functional properties"(Anandharamakrishnan and Ishwarya, 2015).

Encapsulation is a method by which one or many materials kept inside other material. The material is coated with the major material as a liquid, and it is called internal phase, actives, fill, or payload. The material coating is called the wall material, coating or shell (Figure 2.7) (Gibbs, 2009). The wall material used in food products should be food grade materials to provide a barrier between active compounds and harsh environments. This method has a major role in delivering active compounds, for example, it is able to soluble and the controlled release in food products and the gut environment. Two major types of encapsulation are the reservoir and the matrix type (Zuidam and Shimoni, 2010). See Figure 2.8 for details. The reservoir type has a wall material around the active compounds, which is called the capsule, mono, poly, multi-core or core-shell type. The active compounds in the matrix type are dispersed greater than the wall material. Active compounds in the matrix type normally appear at the surface. The standard materials that builds the wall materials is selected depending on the following criteria: (Desai and Park, 2007).

1. Its consistency and rating of reactivity with the active compounds and a harsh environment.

2. The wall materials being certificated as safe for food products or "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) or the European Food Safety Authority (EFSA);

3. Encapsulation cost.

4. Potential of encapsulation.

5. Observance of the final product specifications.

Encapsulation is used in various industries for a wide variety of products (Agnihotri et al., 2012). Different kinds of encapsulation techniques are used in the

food industry including the emulsion technique, spray drying and extrusion (Gibbs, Kermasha, Alli and Mulligan, 1999).

2.8.1 Emulsion techniques

Emulsion techniques use liquids, of which there are three major types to create microcapsules such as emulsion encapsulation, phase separation and extrusion (Bansode et al., 2010). McClements 2005; Appelqvist et al. 2007). These techniques always have two-phase methods: the oil phase and the water phase which are separate. Emulsions are normally produced with high shear including an homogenizer, then stirred with high shear. The encapsulation technique using an emulsions can be applied as a vehicle for the delivery of food products (Appelqvist et a1. 2007). There are two requirements when defining an emulsion for controlling delivery. First, the emulsion must be stable until it reaches the site of food application. Second, the emulsion should be stable for delivery to the wanted organ. Normally, this is the "making and breaking" of emulsions for stability and after delivery. The emulsion techniques of bioactive compounds consist of a core material or dispersed phase in a wall material or a continuous phase, with appropriate emulsifiers such as Tween 80. Thies and Bissery (1984) explained the basic process, of which the major stage is to create a w/o emulsion. The active compound is added to a solution. A small quantity of the aqueous phase is added to a large volume of the oil, then homogenized to create a microcapsule (Krasaekoopt et al., 2003). See Figure 2.9 for details. The microcapsule size is controlled by the speed of the equipment and the size ranges from 25 millimeters. This technique has been applied to the microencapsulation of probiotic bacteria (Lacroix et al., 1990). There are various supporting materials applied with the emulsion techniques including the material of locust bean gum (Audet et al., 1988),

alginate (Sheu and Marshall, 1993), and gelatin (Hyndman et al., 1993; Nawong et al., 2016). Moreover, Nawong and et al. (2016) show that encapsulation in gelatin and maltrodextrin and cross linked by TGase shows a higher survival rate of probiotic *Lactobacillus* sp. 21C2-10 during exposure to gastrointestinal conditions when compared with the free form of the *Lactobacillus* sp.21C2-10. Therefore, this micro capsule that is microencapsulated by the emulsion technique is appropriate to be added to food products for making probiotic food products into functional foods.

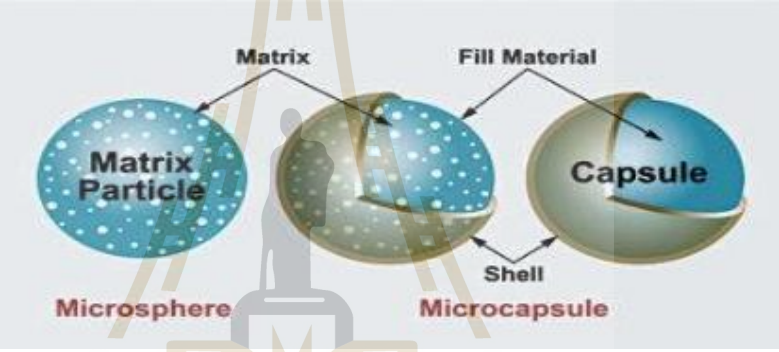

Figure 2.6 Core and shell of microcapsule (Agnihotri et al., 2012).

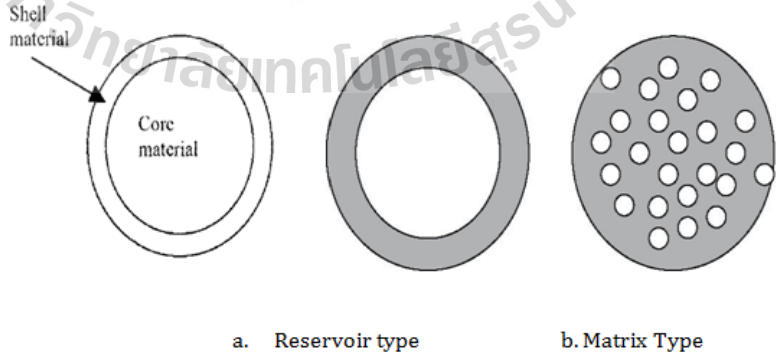

Figure 2.7 Morphology of a microcapsule (Annamalai, 2016).

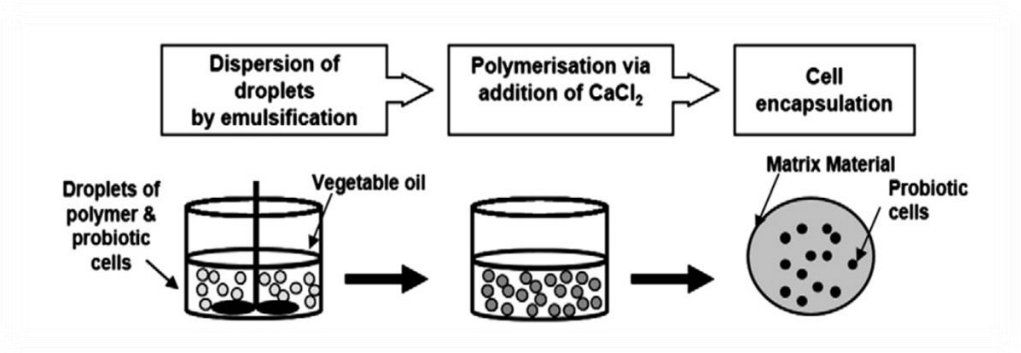

Figure 2.8 Microencapsulation by the emulsion technique. (Krasaekoopt et al., 2003)

2.9 Application of probiotics in food models

Probiotics can be incorporated into food products, such as infant foods, fruit juices, ice-cream, yogurts, and fermented milks. They are normally added at amounts of 10^7-10^9 cfu/g (Dave et al., 1997). The increase in probiotic viability during the storage period and when passing through gastrointestinal tracts are one of the main objectives for the development of food products containing probiotics. The effects on the survival of probiotics in food products is significant in studies and the probiotic delivery to the target organs such as the colon of the consumer. The major factors requiring further investigation concern the survival of probiotics in food products including (Tripathi and Giri, 2014):

1. The physiology of probiotics.
2. The conditions of food manufacturing.
3. The product storage conditions (i.e. temperature)
4. The composition of food products (i.e. acidity, oxygen, moisture)

When developing new products, many researchers are required to confirm that the selected strain will be able to survive well in food products that contain probiotics

and will not affect the sensory quality of food products, such as taste, smell, and texture (Pavli et al., 2018). The probiotic *Lactobacillus* strains are more appropriate for food product development than the *Bifidobacteria* strains (Fijan, 2014). However, many researchers have suggested that the viability of probiotics in free form in food products show a lower survival rate during storage periods and during passage through gastrointestinal conditions (Iravani, Korbekandi and Mirmohammadi, 2014). Moreover, microencapsulation of probiotics improves their viability in the microencapsulation process, food manufacturing, and during storage in food products and gastric acid exposure (Solanki et al., 2013). For example, Sheu and Marshall (1993) suggested that entrapped *Lactobacilli* increased viable probiotic bacteria in ice cream. Homayouni et al. (2008) found that microencapsulated probiotic bacteria could enhance the viable probiotic bacteria in synbiotic ice cream. Magarinos et al. (2007) also found that probiotic ice cream containing encapsulated *Lactobacillus acidophilus* stored at -25^{0}C for 60 days of storage had a percentage survival rate of 87%. Champagne et al. (2015) suggested that microencapsulated *Lactobacillus rhamnosus* had a viable count higher than probiotic bacteria in free forms in ice cream during 210 days of storage period. Moreover, Ribeiro et al. (2014) observed that yogurt containing microencapsulated *Lactobacillus acidophilus* showed more endurance to simulated gastro-intestinal juices than yogurt containing free *Lactobacillus acidophilus*. Further, Matias et al. (2016) reported that ice cream containing free *Lactobacillus acidophilus* LA-5 showed physiological change when stress was induced on the gastro-intestinal tract (in vitro assay). Furthermore, Nawong et al. (2017) suggested that microencapsulated probiotics using maltrodextrin and gelatin as wall materials which had been cross-linked by transglutaminase significantly

enhanced the viability of *Lactobacillus* sp. 21C2-10 after exposure to SGJ and SIJ for 240 mins. at 37^0C. Therefore, it has been well established that microencapsulation techniques protect probiotic bacteria in food products and during passage through gastrointestinal conditions.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Methods

3.1.1 Bacterial strains and culture conditions

Lactobacillus sp. strain 21C2-10 was isolated from cassava pulp from Nakhonratchasima province. Probiotic cells were grown on deMan Rogosa Sharpe (MRS) agar (Himedia, India) at 37ºC for 24 hours under anaerobic conditions and identified using the API 50 CHL (BioMérieux, Marcy-I'Etoile, France) (Nawong, Boonkerd and Oonsivilai, 2013).

3.1.2 Cell culture and growth conditions

Caco-2 cells originated from a human colorectal adenocarcinoma (ATCC Cat. No. HTB-37, ATCC, USA) and prepared following Garrett et al. (1999); Ferruzzi et al. (2001). The Caco-2 cells were grown on complete medium in T-75 flask (75 cm^2 cell culture flask), the complete medium (DMEM, Gibco cat.no. 11965-092) including, 10.0% (v/v) of heat-inactivated fetal bovine serum (FBS, Gibco cat.no. 10270-098), 1% (v/v) of non-essential amino acids (MEM NEAA, Gibco cat.no. 11140-050), L-glutamine (Gibco cat.no. 25030-081 and penicillin-streptomycin (Pen-Strep, Gibco cat.no. 15140-122). Caco-2 cells were incubated at 37ºC, 5% CO_2 and 95% O_2 in a humidified incubator (Panasonic, Japan). Every 2 days, a completely new medium was used.

3.1.3 Adhesion properties

3.1.3.1 Cell adhesion

The cell adhesion of *Lactobacillus* sp. 21C2-10 to the human colorectal cancer cell lines (Caco-2) were assessed following Duary et al. (2011). The Caco-2 cell lines were seeded on the complete medium in 6-well plates for 24 hrs at 37^{o}C, 5% CO_2 in a humidified incubator. The *Lactobacillus* sp. 21C2-10 were diluted in a completely new medium and transferred to each well and incubated at 37^{o}C for 2 hours in 5% CO_2. After that, a sterilized phosphate-buffered saline (PBS) (GIBCO®, cat.no. 70011, Thermo Fisher Scientific Inc.) was rinsed the unadhered *Lactobacillus* sp. 21C2-10. The adhered *Lactobacillus* sp. 21C2-10 were counted on MRS Agar. The adhesion was calculated as follows:

$$\%\text{Adhesion} = (V1 \times 100)/V0$$

Where,

V0 = the number of viable *Lactobacillus* sp. 21C2-10 before being tested.

V1 = the number of viable *Lactobacillus* sp. 21C2-10 which adhered to Caco-2 ce

3.1.3.2 Cell surface hydrophobicity

Cell surface hydrophobicity was assessed through microorganism adherence to hydrocarbons (Duary et al., 2011). Washed *Lactobacillus* sp. 21C2-10 were resuspended in 5 mL of buffer (pH 7.0). The initial absorbance was measured at 600 nm. Hydrocarbon (including hexadecane or isooctane) (1 mL) was added to 5 mL to the suspended probiotic cells and vortexed. After incubation for 1 hr. at 37ºC, a phase separation occurred. The aqueous phase was measured the absorbance at 600 nm and calculated using the formula, when the cell surface hydrophobicity was more

than >70%, between 50-70% and lower than 50% to give the percentage of high, moderate and low hydrophobicity, respectively.

$$\% \text{ cell surface hydrophobicity} = ((1-A1)/A0) \times 100$$

Where,

A0 is the initial absorbance.

A1 is the aqueous phase

3.1.4 Preparation of the secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML)

The secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) were prepared as reported (Haghshenas et al., 2014). *Lactobacillus* sp. 21C2-10 was inoculated in MRS broth at 37°C until the late phase of the exponential phase, then the SML were separated by centrifuging at 4000 x g for 10 min at 4°C, the pH was adjusted to 7.2 with 1 M NaOH and lyophilized using a freeze dryer (Alpha 1-4 LSC plus, Osterode am Harz, Germany). The SML was dissolved and diluted in the complete media, then sterilized using a 0.22 µm filter before being used to treat the cancer cells in further experiments. Furthermore, short chain fatty acid (SCFA) was measured using Gas Chromatography by Suranaree University of Technology.

3.1.5 Cytotoxicity

The effect of secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) on cell viability was evaluated using the MTT assay. The Caco-2 cells were seeded on a complete medium in a 96-well plate for 24 hrs. at 37^{o}C, 5% CO_2 in a humidified incubator. The SML were added with five concentrations at 0, 10, 20, 30 and 40 mg/ml for 24 h. The complete media was removed, 0.5 mg/ml of MTT solution was

added and incubated for 4 hrs, then removed, and the crystal was dissolved in DMSO. The absorbance was measured (570 nm) by a microplate reader (Spectrostar Nano, Victoria, Australia). The absorbance of control was obtained as cell viability (%).

3.1.6 Apoptosis properties

3.1.6.1 Apoptosis assessment by a Tali image based cytometer

The Caco-2 cell lines were seeded on a complete medium in a 6-well plate for 24 hrs at 37°C, 5% CO_2 in a humidified incubator. The SML were added with five concentrations at 0, 10, 20, 30 and 40 mg/ml for 24 h. Caco-2 cells were harvested and washed twice with PBS. Caco-2 cells were stained with a Tail apoptosis Kit (Catalog no. A10788) in the dark room at room temperature for 20 min. Live, apoptotic, and dead cells were analyzed using a Tali image-base cytometer (Invitrogen, Waltham, Massachusetts, USA).

3.1.6.2 DAPI staining

Morphological changes of nucleic acid measured by DAPI staining method. Caco-2 cell lines were grown on a glass coverslip for 24 hrs. at 37°C, 5% CO_2 in a humidified incubator. Caco-2 cell lines were treated with or without SML for 24 hrs. Caco-2 cells were washed with cold PBS (2 times), and fixed with 4% of paraformaldehyde at room temperature for 10 min. The fixed cells were washed with PBS (2 times). The fixed Caco-2 were stained with DAPI solution at a concentration of 1.5 μg/ml and coverslip were placed on a glass slide, then observed by a fluorescent microscope (Bio-rad, Canada).

3.1.6.3 RNA isolation and reverse transcription (RT)-PCR

The Caco-2 cell lines were seeded on a complete medium in a 6-well plate for 24 hrs. at 37°C, 5% CO_2 in a humidified incubator. The SML were added

with five concentrations at 0, 10, 20, 30 and 40 mg/ml for 24 h. Caco-2 cells were harvested and washed with PBS (2 times). Total RNA was isolated using NucleoSpin RNA kit (Macherey-Nagel, Dueren Germany), according to the producer's method. The isolated RNA synthesized complementary DNA (cDNA) using qPCR RT Master mix (Vivantis Technologies Sdn. Bhd, Malaysia), according to the producer's method. The gene expression used qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, Canada), according to the producer's method and was performed using the Biorad/C1000Touch Thermocycle (Biorad, CA, USA) with specific primers. The relative expression levels of mRNAs were determined with the *GAPDH* gene. Primer sequences are shown in Table 3.1.

Table 3.1 Primer sequences.

Gene name	Primer	Primer Sequence (5'-3')
BCL2	Reverse	CCAgAggAggAggTAgggAC
	Forward	TgATgTgAgTCTgggCTgAg
BAX	Reverse	ggAggAAgTCCAATgTCCAg
	Forward	TCTgACggCAACTTCAACTg
P53	Reverse	gCTCgACgCTAggATCTgAC
	Forward	gCTTTCCACgACggTgAC
CASP3	Reverse	gAgTCCATTgATTCgCTTCC
	Forward	ggATgggTgCTATTgTgAgg
CASP8	Reverse	TgTCCAgTTgTTCCCCAATA
	Forward	ggTCACTTgAACCTTgggAA
CASP9	Reverse	AggTCCTCAAACCTTCCTgg
	Forward	gTggACATTggTTCTggAgg

3.1.7 Probiotic encapsulation

3.1.7.1 Bacterial strains and culture conditions

Lactobacillus sp. strain 21C2-10 was isolated from cassava pulp in Nakhonratchasima province. Probiotic bacteria were identified by Nawong et al. (2013).

3.1.7.2 Preparation of activated probiotic cultures

The frozen stock culture of probiotic bacteria was stored at -20^{0}C. 1 ml of the stock cultures was inoculated with MRS broth (Himedia Ltd.). The probiotic bacteria were incubated at 37^{0}C for 24 hrs until the the late phase of the exponential phase. Probiotic bacteria was harvested using centrifugation at 3578 × g at 4^{0}C for 10 mins (Thermo Scientific Ltd., Asheville, NC, USA) then washed with 0.1% (w/v) peptone salt solution (Himedia Ltd.) (2 times) and separated. A cell pellet used in the microencapsulation process.

3.1.7.3 Preparation of microencapsulated probiotic

The probiotic bacteria was microencapsulated by an emulsion technique using a modified microencapsulation method reported by Nawong et al. (2016). The stock solutions of 24% (w/v) gelatin (Sigma-Aldrich Ltd., Saint Louis, MO, USA) and 24% (w/v) maltodextrin (Sigma-Aldrich Ltd.) were separately added into 0.5% (w/v) NaCl (Carlo Erba, Val de Reuil, France) and stirred at 45^{0}C for 20 mins. Cell pellet (1 g) was added into the 24% (w/v) maltodextrin with the ratio of probiotic bacteria to maltodextrin being equal to 15:1. Then, gelatin was added into the cell-maltodextrin mixture at a ratio of 2:1. Ten units of TGase (Ajinomoto Ltd., Paris, France) per g of gelatin (Unit/g) were added into the cell-maltodextrin-gelatin mixture. Then, the mixture was emulsified in oil containing Span 85 (0.5 %w/w) of

(Sigma-Aldrich Ltd.) (aqueous: oil; 1:5) and stirred at 900 rpm at room temperature (22-23^{0}C) for 18 hrs. using a magnetic stirrer. The microencapsulated probiotic bacteria was washed with 0.85% (w/v) of NaCl and 0.5% (w/v) of Tween 80 (Sigma-Aldrich Ltd.), respectively. The microencapsulated probiotic bacteria were harvested using centrifugation at 500 × g at room temperature for 1 min and stored at 4^{0}C.

3.1.8 Physicochemical properties of microencapsulated probiotics.

3.1.8.1 Proximate analysis

3.1.8.1.1 Moisture content (AOAC, 925.10)

The samples were put into dried moisture cans and dried in a hot air oven (Memmert, Germany) at 105ºC for 24 h, then transferred to a desiccator to cool and then reweighed until the weight was constant. The moisture content of the sample was calculated as:

$$\text{Moisture (\%)} = ((M0-M1)/M0) \times 100$$

M0 = weight of sample before drying (g)

M1 = weight of sample after drying (g)

3.1.8.1.2 Ash determination (AOAC, 900.02A)

The crucibles were burned in the muffle furnace at 550ºC for 3h and transferred to the desiccator for cooling. The sample was put in a burned crucible and placed into a muffle furnace at 550ºC for 18 h, then transferred to a desiccator to cool and reweighed until the weight was constant. The ash (%) of the sample was calculated as:

$$\text{Ash (\%)} = A1/A0 \times 100$$

A1 = Ash weight (g)

A0 = Sample weight (g)

3.1.8.1.3 Crude protein determination (AOAC, 928.08)

Crude protein was determined by the Kjeldahl method (AOAC, 2005). The sample was weighed into a Kjedahl digestion flask and 25 ml of sulphuric acid, 0.5 g of copper sulphate and 5 g of potassium sulfate were added. The mixture was digested until clear. The digest was mixed with 40% (w/v) of NaOH and distilled in a distillation apparatus for 5 minutes. The released ammonia was trapped with 25 ml of 4% boric acid containing a mixed indicator and titrated with HCl. The crude protein (%) calculated by using the formula below.

$$\text{Crude protein (\%)} = (N \times V \times 0.014 \times F \times 100) / \text{Sample weight (g)}$$

N = concentration of HCl (mol)

V = the volume of HCl (ml)

F = 5.55

3.1.8.1.4 Crude fat determination (AOAC, 963.15)

The amount of crude fat was determined using AOAC (2005). The sample was weighed on filter paper and put into a cellulose thimble. The weighed samples were extracted in the extractor (Foss tecator soxtec avanti 2050, Hoganas, Sweden) for 4 h with 80 ml of petroleum ether. The extracted samples were placed in an oven at 105ºC for 24 h, then transferred to a desiccator to cool and reweighed until the weight was constant. The crude fat (%) of the sample was calculated as:

$$\text{Crude fat (\%)} = (F0-F1) / F0 \times 100$$

F0 = weight of sample (F0)

F1 = weight of sample after extraction (g)

3.1.8.1.5 Crude fiber determination (AOAC, 978.10)

The sample from 3.2.8.2.4 was used to determine the crude fiber. 150 ml of 1.25% sulphuric acid was added to the samples and boiled for 30 min, then the samples were filtered and washed with boiling water until the filtrate was neutral. 1.25% (w/v) of NaOH (150 mL) were added and boiled for 30 min, then the samples were filtered and washed with boiling water until the filtrate was neutral and washed twice with 95% ethanol and transferred to a crucible, then dried at 105ºC in the hot air oven until a constant weight was obtained. The crucible was cooled in a desiccator and weighed. The crucible was burned in a muffle furnace (Gallenkamp muffle furnace, England) at 550ºC for 2 h. The crucible was transferred into desiccator to cool and then reweighed until a constant weight was obtained. The crude fiber (%) of the sample was calculated as:

$$\text{Crude fiber (\%)} = \text{CF1/CF0} \times 100$$

CF1 = Weight of fiber (g)

CF0 = Sample weight (g)

3.1.8.1.6 Determination of carbohydrates

The available carbohydrate was calculated by the differences between the amounts of moisture, ash, crude fat, crude protein and dietary fiber:

$$\text{Available carbohydrate (\%)} = 100 - (\text{Moisture (\%)} + \text{Ash (\%)} + \text{Crude fat (\%)} + \text{Crude protein (\%)} + \text{Crude fiber (\%)})$$

3.1.8.2 Particle size measurement

The particle size of the microencapsulated *Lactobacillus* sp. 21C2-10 was measured and the diameter was found to be 600 microcapsules at 1000X using an optical microscope (Carl Zeiss, Germany) with a calibrated micrometer scale and the structure of the microencapsulated probiotic was examined with a optical microscope.

3.1.9 Survival of probiotic in free and microencapsulated forms during exposure to gastro-intestinal conditions

3.1.9.1 Preparation of simulated gastric juice (SGJ) and simulated intestinal juice (SIJ)

Simulated gastric juice (SGJ) was freshly prepared using a method reported by Nawong et al. (2016). SGJ was prepared by adding 0.3 g of pepsin (Sigma-Aldrich Ltd.) to 0.2% (w/w) NaCl. The volume was adjusted to 1000 mL. The pH was adjusted to 2.0 and sterilized.

Simulated intestinal juice (SIJ) was freshly prepared using a method reported by Nawong et al. (2016). SIJ was prepared by adding 8 g of pancreatin (Sigma-Aldrich Ltd.) and 36 g of bile salts (Fisher Scientific Ltd.) to 0.02M phosphate buffer. The volume was adjusted to 1000 mL. The pH was adjusted to 7.4 and sterilized.

3.1.9.2 Survival of probiotics in free and microencapsulated froms during exposure to simulated gastric juice

Free cells and microencapsulated probiotics (0.5g) were added to 4.5 mL of SGJ, mixed and sealed with parafilm then incubated for 30, 60, 120 mins at 37^0C. Survival of probiotic bacteria were enumerated. The microencapsulated probiotic bacteria sample was released from the microcapsules according to the

method proposed by Nawong et al. (2016). 4.5 g of 0.5mM CaCl2 (Fisher Scientific Ltd., Ottawa, Ontario, Canada) containing 40 unit/ml of collagenase (Sigma-Aldrich Ltd.) were added. In addition, pH was adjusted to 7.4 and stirred for 10 secs, then the ice cream sample was incubated at 37^0C for 1 hr. The probiotic bacteria were enumerated on MRS agar and incubated under anaerobic conditions at 37^0C for 48 hrs. The survival of viable probiotic bacteria was reported according to the survival rate (%), which was calculated using the following formula:

$$\%\text{Survival rate} = (\log Nt/\log N0) \times 100$$

Log Nt is the viability of probiotic bacteria at time t (log CFU/g).

Log N0 is the viability of probiotic bacteria before treated (log CFU/g).

3.1.9.3 Survival of probiotics in free and microencapsulated forms during sequential exposure to simulated gastro-intestinal juice

Free cells and microencapsulated probiotics (0.5g) were added to 4.5 mL of SGJ, mixed and sealed with parafilm, and incubated for 60 mins at 37^0C. 25 mL of SIJ tempered at 37^0C were added to the mixtures and the pH was adjusted to 7.4. The mixture was adjusted to 10 mL with phosphate buffer and sealed with parafilm and incubated for 60, 120, 180 and 240 mins, respectively, at 37^0C. Survival of probiotic bacteria was enumerated as described in Section 3.1.9.2. The survival of viable probiotic bacteria was reported according to the survival rate (%), which was calculated following formula in Section 3.1.9.2.

3.1.10 Probiotic ice cream

3.1.10.1 Preparation of ice cream

Ice cream was prepared using a modified method reported by Marshall (2003). All ingredients were weighed separately. Skimmed milk powder (Miky SMP Co., Wellington, New Zealand) was dissolved in water, then heated up to 45^0C. Dry ingredients, such as sucrose (Mitrphol Ltd., Khonkaen, Thailand) and a stabilizer (Palsgaard Ltd., Juelsmide, Denmark) were dissolved in the SMP solution, then the temperature was increased to 75^0C. The butter fat (Elle&Vire Ltd., France) was added to the mixture, then pasteurized at 75^0C for 15 mins. The emulsifier (Tween 80, Sigma-Aldrich Ltd.) was added to the mixture. The mixture was stirred and homogenized under pressure at 2500 and 5000 psi (APV Gaulin Co., Inc., Lawrence, USA) and cooled down to 4^0C in the refrigerator (PTV 19T/43, Montecchio, Italy), then incubated in the refrigerator for 12 hrs. Furthermore, there were 3 treatments for probiotic inoculation as follows: 1) no added probiotic bacteria, 2) 1 g of free *Lactobacillus* sp. 21C2-10 was added, and 3) 1 g of microencapsulated *Lactobacillus* sp. 21C2-10 was added. After that, all the treated ice cream samples were mixed with a hand blender (Taylor Ltd., Rockton, Illinois, USA) for 15 mins. The ice cream samples were packed in plastic cups at 50 g per cup. The ice cream samples were hardened and storage in a freezer (Kendro Laboratory Products Ltd., Asheville, USA) at -20^0C for 180 days. The formulations of all treatments are shown in Table 3.2.

3.1.10.2 Enumeration of probiotic bacteria in ice cream

The viability of probiotic bacteria was determined in ice cream samples during frozen storage at -20^0C for 180 days. The microencapsulated probiotic

in ice cream samples were released from the microcapsules according to the method proposed by Nawong et al. (2016). 10 g of the ice cream sample were re-suspended in 90 ml of 0.5mM $CaCl_2$ (Fisher Scientific Ltd., Ottawa, Ontario, Canada) containing 20 unit/ml of collagenase (Sigma-Aldrich Ltd.). In addition, the pH was adjusted to 7.4 and stirred for 10 secs, then the ice cream sample was incubated at 37^0C for 1 hr. The probiotic bacteria were enumerated on MRS agar and incubated under anaerobic conditions at 37^0C for 48 hrs. The survival of viable probiotic bacteria was reported according to the survival rate (%), which was calculated using the following formula in Section 3.1.9.2.

Table 3.2 The formulations of all treatments.

Composition (g)	**T1**	**T2**	**T3**
Butter fat	300	300	300
Skimmed milk powder	330	330	330
Sucrose	300	300	300
Stabilizer	10.5	10.5	10.5
Tween 80	4.5	4.5	4.5
Water	2055	2055	2055
Free cells of *Lactobacillus* sp. 21C2-10 (about 10 log CFU/g)	-	30	-
Microencapsulated cells of *Lactobacillus* sp. 21C2-10 (about 10 log CFU/g)	-	-	30

3.1.10.3 Proximate analysis

The ice cream samples were analyzed for chemical composition after one day of storage (-20^{o} ± 10^{o}C) following the AOAC (2005) official protocols. Moisture content (AOAC, 925.10), ash (AOAC, 900.02A), protein (AOAC, 928.08), fat (AOAC, 963.15), crude fiber (978.10) and carbohydrate were determined by theirs differences.

3.1.10.4 The pH value and titratable acidity of ice cream during storage time

The acidity (expressed as % lactic acid) was determined by using a standardized solution of 0.01M NaOH (Carlo Erba Ltd.) and taking 10 g of the ice cream sample, adding 1 ml of phenolphthalein indicator and then titrated with NaOH until the light pink is durable. The ice cream pH value was measured using a pH meter (SI Analytics Ltd., Weilheim, Germany).

3.1.10.5 Microbial spoilage analysis

The total aerobic count, Coliforms and Escherichia coli were determined using Petrifilm Aerobic count plateTM and Petrifilm EC™ Count Plates (3M petrifilm Co., Saint Paul, Minnesota, USA) respectively, and incubated at 37^{0}C for 24 hrs. For yeast and mold enumeration, the ice cream samples were spread on Potato Dextrose Agar (PDA) (Himedia Ltd.) and incubated at 30^{0}C for 72 hrs.

3.1.10.6 Sensory evaluation

The sensory evaluation of the ice cream samples after 1 and 180 days storage at -20ºC were conducted using a 9-point hedonic scale of appearance, flavor, texture and total acceptability (1=dislike extremely and 9=like

extremely). A panelist group of 30 untrained panelists (18-35 years old) were students of the Food Technology Department, Suranaree University of Technology, Nakonratchasima, Thailand. Approximately 25 g of each ice cream sample were coded with a three-digit randomized number and served to the panelists.

3.1.11 Survival of probiotic in free and microencapsulated forms in ice cream during exposure to gastro-intestinal conditions.

3.1.11.1 Preparation of simulated gastric juice (SGJ) and simulated intestinal juice (SIJ)

Simulated gastric juice (SGJ) was freshly prepared using a method reported by Nawong et al. (2016). SGJ was prepared by adding 0.3 g of pepsin (Sigma-Aldrich Ltd.) to 0.2% (w/w) NaCl. The volume was adjusted to 1000 mL. The pH was adjusted to 2.0 and sterilized.

Simulated intestinal juice (SIJ) was freshly prepared using a method reported by Nawong et al. (2016). SIJ was prepared by adding 8 g of pancreatin (Sigma-Aldrich Ltd.) and 36 g of bile salts (Fisher Scientific Ltd.) to 0.02M phosphate buffer. The volume was adjusted to 1000 mL. The pH was adjusted to 7.4 and sterilized.

3.1.11.2 Survival of probiotic in free and microencapsulated from in ice cream during exposure to simulated gastric juice

Ice cream samples (25 g) were added to 225 mL of SGJ, mixed and sealed with parafilm then incubated for 30, 60, 120 mins at 37^0C. The survival of the probiotic bacteria were enumerated on the MRS agar as described in Section 2.5. The survival of viable probiotic bacteria was reported according to their

survival rate (%), which was calculated following the formula given in Section 3.1.9.2.

3.1.11.3 Survival of probiotic in free and microencapsulated probiotic bacteria in ice cream during sequential exposure to simulated gastro-intestinal juice

Ice cream samples (25 g) were added to 225 mL of SGJ tempered at 37^0C, mixed and sealed with parafilm and incubated for 60 mins at 37^0C. 25 mL of SIJ tempered at 37^0C were added to the mixtures and the pH was adjusted to 7.4. The mixture was adjusted to 500 mL with a phosphate buffer and sealed with parafilm and incubated for 60, 120, 180 and 240 mins at 37^0C. The survival of the probiotic bacteria was enumerated as described in Section 3.1.10.2. The survival of the viable probiotic bacteria was reported according to the survival rate (%), which was calculated by using the formula in Section 3.1.9.2. Furthermore, the structure of the microcapsules in the ice cream during exposure to gastrointestinal conditions were observed using an optical microscope.

3.1.11.4 The morphology of probiotic bacteria in ice cream samples during sequential exposure to simulated gastro-intestinal juices

Briefly, the samples were received from the in vitro simulated gastrointestinal conditions assay (Section 3.1.11.3); ice cream containing microencapsulated probiotic bacteria was released by collagenase as described in Section 3.1.10.2, then centrifuged (5000 × g for 5 mins) to separate the cell pellets and were re-suspended in 0.85% (w/v) NaCl. The cell suspensions were centrifuged

and fixed with 2% (w/v) glutaraldehyde (Carlo Erba Ltd.) for 24 hrs at 4^0C. The samples were washed three times with a buffer for 15 mins, post-fixed with 2% (v/v) osmium tetroxide (Carlo Erba Ltd.) for 3 hrs and then washed three times with distilled water for 15 mins. The samples were dehydrated in ethanol solution at concentrations of 50%, 70%, 90% and finally with 99.5% (v/v) ethanol (Carlo Erba Ltd.). The samples were dropped into a cover slide and air-dried at room temperature. The dried cells were placed on aluminum stubs and coated with gold and then observed using a scanning electron microscope (Auriga Ltd., Frankfurt, Germany) at 2.5 kV. Sample images were captured at 10000X.

3.1.12 Statistical analysis

All experiments were conducted in triplicate (n = 3). The results were statistically analyzed using SPSS (version 16.0, SPSS Inc., USA). The data is shown as mean ± standard deviation (SD). The group means were compared by using the Tukey post hoc test at 5% levels of significance.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Adhesion properties

4.1.1 Cell adhesion and cell surface hydrophobicity

The properties of cell adhesion to the cell surfaces of the epithelial cells and colonization has been one of the probiotic properties for selected probiotic strains (Collado et al., 2007). The cell adhesion of *Lactobacillus* sp. 21C2-10 to Caco-2 human colorectal adenocarcinoma cells was investigated. *Lactobacillus* sp. 21C-10 ($5.6x10^4$ CFU/well) adhered to Caco-2 cells to the number of 14.44±0.58%. Moreover, the physicochemical properties in the cell surface of probiotic bacteria affect the attachment of the epithelial cells. Furthermore, the cell surface hydrophobicity of *Lactobacillus* sp. 21C2-10 was adhered with hexadecane (91.56±0.39%) and isooctane (77.95±0.39%). Since the physicochemical properties of the cell surface of bacteria cells include a peptidoglycan layer, polysaccharides and cell surface proteins in the cell surfaces which are connected with the cell binding properties of the polysaccharides and glycoprotein of the epithelial cells (Sánchez et al., 2008; Veléz et al., 2007). However, the adhesion of probiotic cells is related to a variety of biochemical properties of probiotic bacteria and epithelial cells, including hydrophobicity, electrostatic interactions and cellular structures (Servin and Coconnier, 2003). It affects *Lactobacillus* sp. 21C2-10 which show cell adhesion

properties and cell surface hydrophobicity. The adhesion properties of *Lactobacillus* sp. 21C2-10 in the Caco-2 cell leads to the colonization of *Lactobacillus* sp. 21C2-10.

Table 4.1 Cell adhesion and cell surface hydrophobicity of *Lactobacillus* sp. 21C2-10.

Adhesion to	**Cell surface hydrophobicity**	
Caco-2 (%)	**% adhesion to hexadecane**	**% adhesion to isoactane**
14.44±0.58	91.56±0.39	77.95±0.39

Values are represented by means ± standard deviation (n = 3).

4.2 Cytotoxicity

Cytotoxicity was evaluated by the secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) on Caco-2 cancerous human cell lines when using the MTT assay as a colorimetric method for the evaluation of cell viability. The dosage of SML on cytotoxic effects of Caco-2 cells were evaluated at concentrations of 10, 20, 30 and 40 mg/ml. The cell viability of Caco-2 cells decreased when with increasing doses of SML (Figure 4.1). The SML caused a significant ($P < 0.05$) decrease in cell viability (%) in a dose-dependent manner. However, the most anti-proliferative effect was represented in the highest doses. The cell viability of Caco-2 cells were 88.29±5.62%, 64.33±2.67%, 52.59±5.63%, and 28.74±0.42%, respectively. This result shows that the anti-proliferative effect of SML on Caco-2 cancer cells had significant differences with control (untreated) and MRS-treated groups. On the other hand, the effect of the MRS-treated group showed no effect on the anti-proliferative effects of Caco-2 cell lines. The recent research has emphasized the anti-cancer properties of colon cancer cell lines by probiotics (De Moreno De Leblanc and Perdigon, 2010). Similarly, the

results reported by Nami et al. (2014) suggest that the metabolites secreted from *Lactobacillus* sp. have the most potent anti-proliferative effects or cytotoxicity affects the inhibition of human colorectal cancer cells (HT-29). Haghshenas et al. (2014); Haghshenas et al. (2015); Saxami et al. (2016); and Orlando et al. (2016) suggest that bacterial secreted metabolites from probiotic bacteria have anti-proliferative effects or cytotoxicity effects against human gastrointestinal cancer cells by induction of apoptosis. In addition, secreted metabolites from probiotics such as SCFA show anti-cancer effects via apoptosis induction and activated Caspase-3 in human colon cancer cell lines (Femia et al., 2002; Kim et al., 2007). Moreover, Escamila, Lane and Maitin (2012) have shown that secreted metabolites from *Lactobacillus* sp. could decrease the activity of matrix metalloproteinase-9 which is related to the apoptosis pathway. Gonet et al. (2007) and Thirabunyanon et al. (2009) show secreted metabolites from probiotic bacteria decrease the growth of colon cancer cell lines through apoptosis induction. Normally, it is believed that SCFA including butyric, acetic and propionic acid show anti-proliferative effects (Thirabunyanon and Hongwittayakon, 2012). Choi et al. (2006) show that secreted metabolites from *Lactobacillus acidophilus* could inhibit proliferative effects of colon cancer cell lines. Thus, the SML contained SCFA (1.60 mmol/g of SML) including acetate (1.59 mmol/g of SML) and propionate (0.008 mmol/g of SML) which is related to the cytotoxicity of Caco-2 cell lines. These results indicate that secreted metabolites of *Lactoacillus* sp. 21C2-10 may have efficient anticancer properties against Caco-2 cancerous cells.

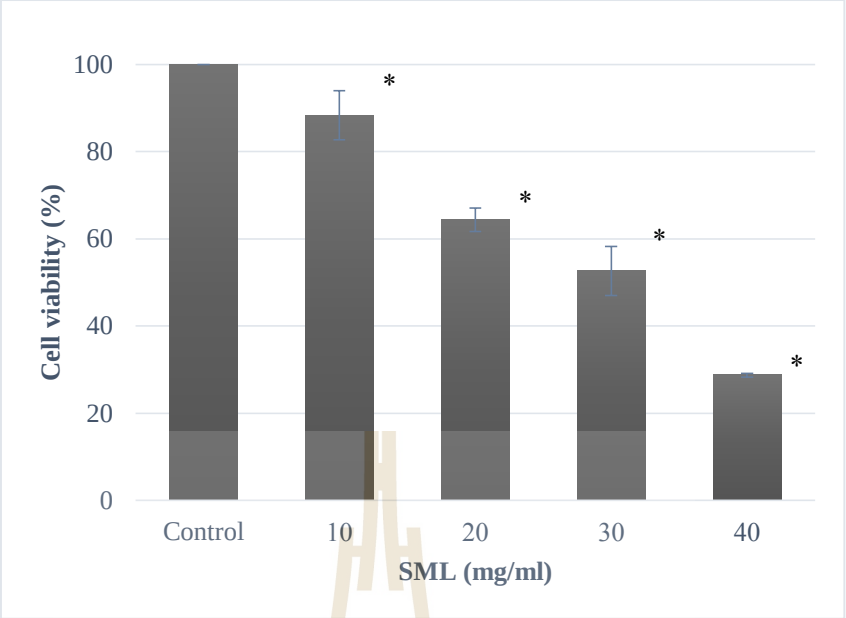

Figure 4.1 Secreted metabolites from *Lactobacillus* sp. 21C2-10 (SML) inhibited cell viability of human colorectal cancer cells (Caco-2) when treated with different concentrations of SML (0, 10, 20, 30 and 40 mg/ml) for 24 hours and assessed cell viability by MTT assay. Values are expressed as mean ± SD (n=3)

*($p<0.05$) versus control cells

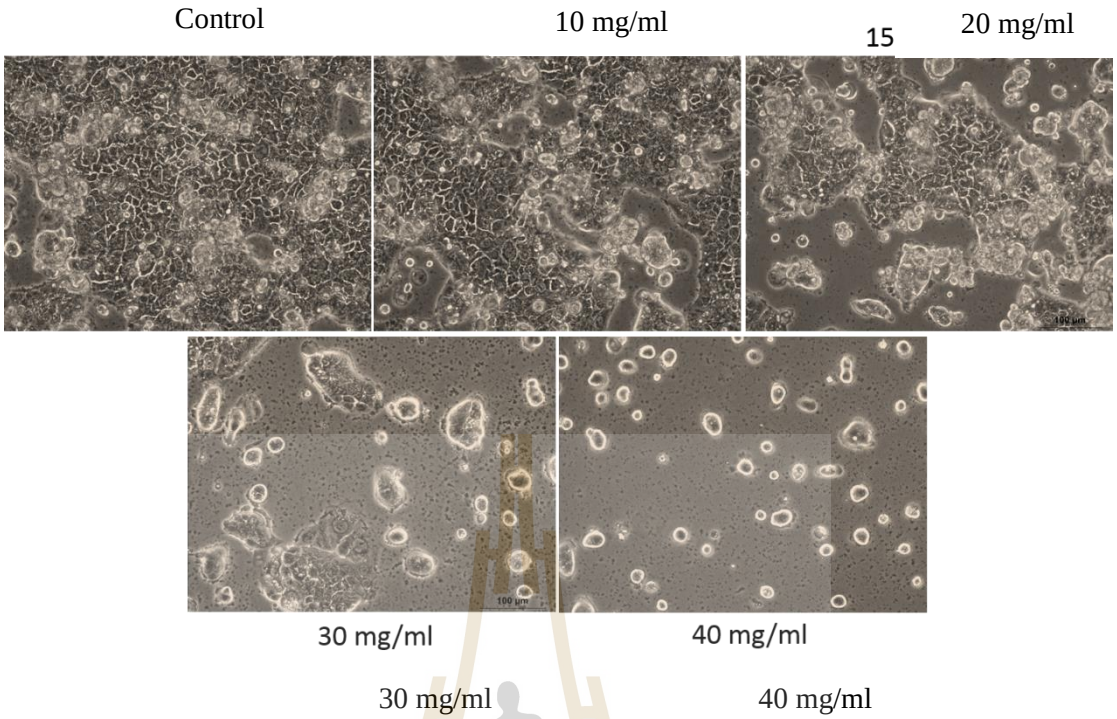

Figure 4.2 Cellular morphology of Caco-2 cells treated with secreted metabolites of *Lactobacillus* sp. 21C2-10 at different concentrations of SML (0, 10, 20, 30 and 40 mg/ml) for 24 hours observed under phase contrast microscopy.

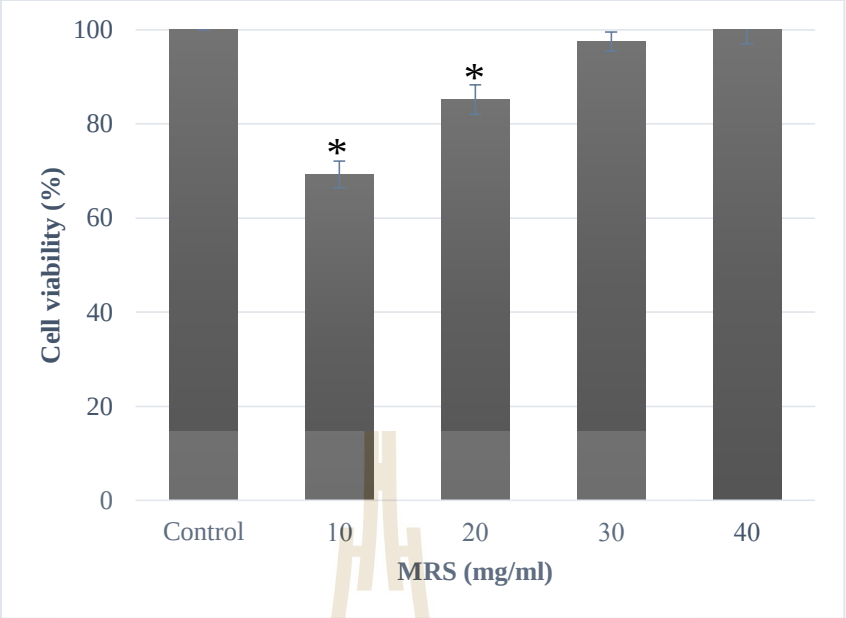

Figure 4.3 MRS broth inhibited cell viability of human colorectal cancer cells (Caco-2) when treated with different concentrations of MRS broth (0, 10, 20, 30 and 40 mg/ml) for 24 hours and assessed cell viability by MTT assay. Values are expressed as mean ± SD (n=3).

*($p<0.05$) versus control cells

4.3 Apoptosis properties

4.3.1 Apoptotic assessments by Tali image base cytometer

A tali image base cytometer verified the secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptosis on Caco-2 cancer cells. Staining of annexin V and propidium iodide was used to detect both apoptotic and necrotic or dead cells. Apoptotic cells show as green, dead or necrotic cells show as red and green (noted as yellow) under fluorescence light. The results show that SML significantly increased apoptotic cells (%) compared with the control group (Fig. 4.4). The treated cells (40 mg/ml) showed live, dead and apoptotic cells (%) of 26.33±4.62%,

2.00±1.00% and 71.67±4.73%, respectively. The treated cell lines represent apoptotic cells in a dose-dependent manner. The treated Caco-2 cells show the live cells (%) decreased from 86.33±3.79% to 26.33±4.62%, and the apoptotic cells (%) increased from 12.00±3.46% to 71.67±4.73% when the dose of SML was increased. Similar results were reported by Haghshenas et al. (2015); Haghshenas et al. (2014) which suggests that cell free culture supernatant (CFCS) from probiotic bacteria induces apoptosis on gastrointestinal human cancer cell lines observed using a flow cytometer which indicated 33%-59.57% of apoptotic cells in which apoptosis could have occurred in the cancer cells more than necrosis. The mechanisms for the inhibition of cancer cell lines by probiotics are unclear (Amir, Beitollah, Zeinab and Ahmad, 2016). However, Probiotics reduce pro-carcinogenic compounds and SCFA binding via active proteins which induced an apoptosis pathway (De LeBlanc, Bibas Bonet, LeBlanc, Sesma, and Perdigón, 2010). Necrosis acts as a subsidiary that induces oxidative stress and secretion of pro-inflammatory cytokines have an influence on the surrounding tissues (Anderson et al., 2002). On other hand, apoptosis is a controlled happening with lowest membrane integrity loss until the late stage, when this mode encompasses phagocytosis through macrophages and induces secretion of anti-inflammatory cytokines (Fadok et al., 1998). This method proves differentiation of healthy viable cells, apotosis cells and necrotic cells. These results show the secreted metabolites from *Lactobacillus* sp. 21C2-10 affect cell deaths in Caco-2 cell lines by apoptosis rather than necrosis.

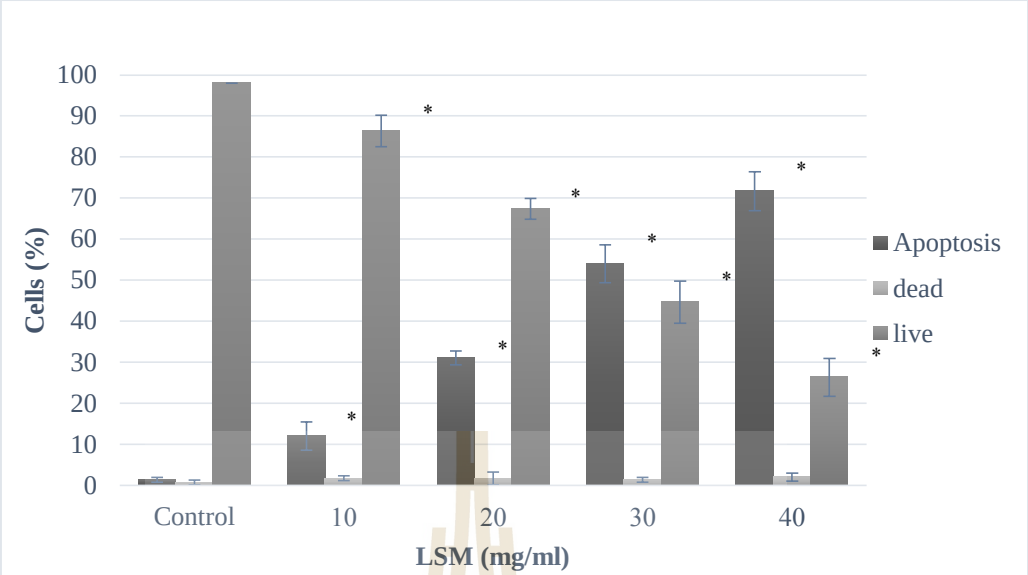

Figure 4.4 Secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptotic cells of human colorectal cancer cells. Cell apoptosis, dead, live (%) was determined by Annexin /PI analysis. Caco-2 were treated with different concentrations of SML (0, 10, 20, 30 and 40 mg/ml) for 24 hours. Values are expressed as mean ± SD (n=3).

*($p<0.05$) versus control cells

4.3.2 DAPI staining

The Caco-2 cancer cell lines were stained and analyzed by a fluorescence microscope. This method is used to evaluate morphological changes of nucleic acids (Savitskiy, Shman, and Potapnev, 2003). The normal cells feature a blue circular, an integral and a clear border. On the other hand, the apoptotic cells feature blue condensed chromosomes and the border of the nucleus is abnormal. There were effects of SML on the morphological changes of nucleic acid of Caco-2 cancer cells when they were exposed to 10, 20, 30 and 40 mg/ml. The results show that the treated

Caco-2 cells represent cell apoptosis, including membrane blebbing, cell shrinking and condensed chromatin and apoptotic bodies. The cell shrinking was found in Caco-2 cells treated with SML (10 mg/ml), condensed chromatin and membrane blebbing were found in Caco-2 cells treated with SML (20 and 30 mg/ml) and apoptotic bodies were found in Caco-2 cells treated with SML (40 mg/ml). In contrast, no apoptotic signals were represented in the untreated Caco-2 cells (Fig. 4.5). Similarly, Jan et al. (2002) reported that bacterial culture supernatants from propionibacterium effects the chromatin condensation of Caco-2 cell lines. Haghshenas et al. (2014) suggested that bacterial secreted metabolites from probiotic bacteria on AGS cells showed apoptotic events. Nami et al. (2014) demonstrated that cell free culture supernatant (CFCS) from *Lactobacillus acidophilus* on Hela cells induced formation of micro nucleic, cell shrinkage, membrane blebbing, nucleus fragmentation and apoptotic bodies. Haghshenas et al. (2015) noted that secreted metabolites of acetic acid bacteria from traditional dairy microbiota on AGS cells showed fragmented or condensed nucleus, membrane blebbing, cell shrinkage and apoptotic body formation. Apoptosis reveals a procedure leading to the death of cells. This apoptosis pathway is physiological, controlling genes, and the progress of tissue and homeostasis, while the apoptotic cells present morphological changing, including membrane blebbing, nuclear condensation, and the formation of apoptotic bodies. Apoptosis proceeds through a series of biochemical events different from those observed in the necrotic process (Jan et al., 2002). DAPI staining is one of the appropriate methods to evaluate morphological change in cell membranes and chromatin in treated cell lines (Savitski et al., 2003). These results prove that the apoptotic pathway leads to cell membrane blebbing, cell

shrinking and condensed chromatin and apoptotic bodies can be regarded as the main cytotoxic properties of secreted metabolites of *Lactobacillus* sp. 21C2-10.

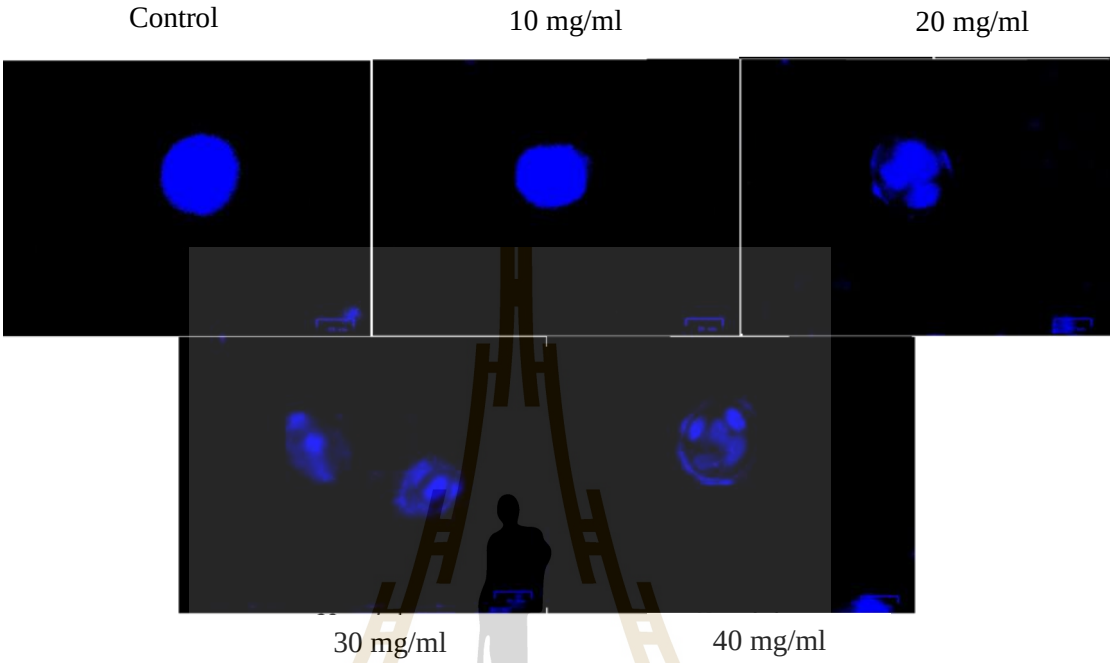

Figure 4.5 Secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptotic cells of human colorectal cancer cells. The nuclear structure of Caco-2 was analyzed via a fluorescence microscope. The cells were fixed and stained with DAPI solution for 10 minutes at room temperature.

4.3.3 RNA isolation and reverse transcription (RT) -PCR

Anti-proliferative properties are often discovered related to the regulation of the gene expression of pro and anti-apoptotic proteins (Bishayee et al., 2013). The gene expression levels were assessed by RT-PCR analysis (Fig. 4.6, 4.7, 4.8). These results indicate that LSM induced an apoptotic pathway in Caco-2 cells. The gene expression of *BAX*, *P53*, *Caspase-3*, *Caspase-8* and *Caspase-9* in Caco-2

cancer cell lines increased significantly when the SML dose was increased. The gene expression of *BAX, P53, Caspase-3* of treated Caco-2 (30 and 40 mg/ml) showed significantly higher genes than control ($p<0.05$). The gene expression of Caspase 8 and 9 of treated Caco-2 (10, 20, 30 and 40 mg/ml) showed significantly higher genes than control ($p<0.05$). However, it decreased the gene expression of *BCL-2* significantly when compared with the control (Fig. 4.6), while the gene expression of *BCL-2* of treated Caco-2 showed significantly lower genes than control ($p<0.05$), suggesting that the induction of apoptosis had occurred by the exposure of secreted metabolites *Lactobacillus* sp. 21C2-10. The SML led to an increase of gene expression of *BAX/BCL-2* ratio in treated Caco-2 colon cancer. Moreover, the gene expression of pro-apoptotic genes was increased.

Caco-2 colon cancer. Moreover, the gene expression of pro-apoptotic genes was increased.

Caspases are a major group that perform in a cascade triggered by extrinsic and intrinsic apoptotic stimuli. *Caspase-9* is the first major protein of caspase that activates the activity of *Caspase-3*. The represent of *Caspase-3* gene is related to the induction of apoptotic pathways in Caco-2 cells via the mitochondrial pathways. *Caspase-3* has been described as an effector caspase associated with the beginning of the death cascade and a major marker of cells provided to the apoptotic signaling pathway. Moreover, the *Caspase-8* gene expression activates the effector of *Caspase-3*, and *Caspase-7*. *Caspase-8* also activates the *BAX* and leads to the release of cytochrome c (Kataoka et al., 1998). Furthermore, cytochrome c released from mitochondria during apoptosis events is related to the permeability of the mitochondrial membrane by the *BCL-2* and *BAX* (Tsujimoto and Shimizu, 2000). The

results show the expression of *BAX* genes in mitochondria which may be the effect of the cytochrome c release leading to an apoptotic pathway (Salvesen and Renatus, 2002). Moreover, *P53* has a major function in reducing the expression of the *BCL-2* group (Schuler and Green, 2001). In all cases, apoptotic pathways were induced via the intrinsic and extrinsic pathways as the Bcl-2 gene expression decreased and *BAX*, *P53*, *Caspase-3*, *Caspase-8* and *Caspase-9* expression increased. These results confirm that the induction of apoptotic pathways included intrinsic and extrinsic apoptotic pathways in Caco-2 colon cancer administered with secreted metabolites of *Lactobacillus* sp. 21C2-10.

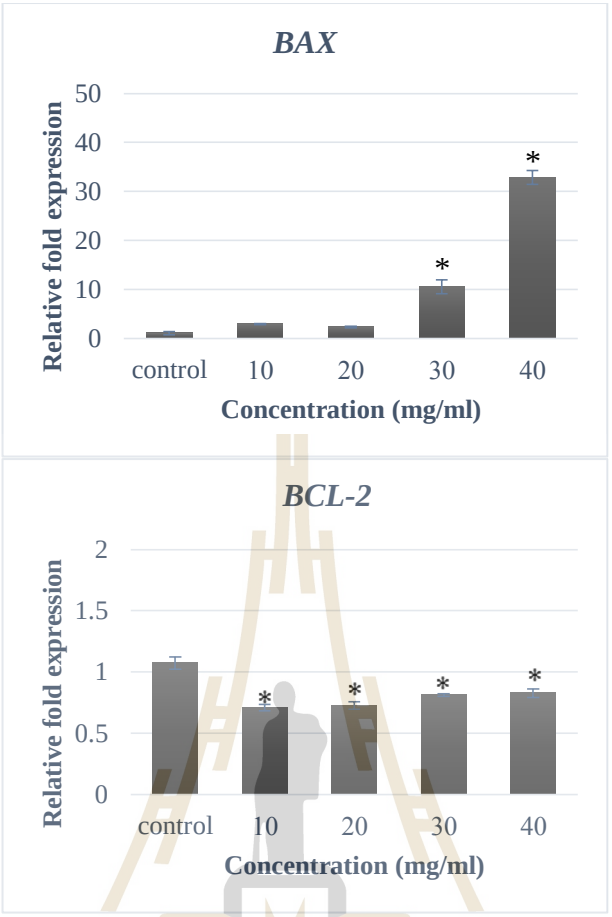

Figure 4.6 Secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptotic cells of human colorectal cancer cells. The mRNA expression level of apoptotic genes, *BAX* and *BCL-2* were evaluated by semi-quantitative RT-PCR to present the relative expression of *BAX* and *BCL-2* genes. Values are expressed as mean ± SD (n=3).

*($p<0.05$) versus control cells.

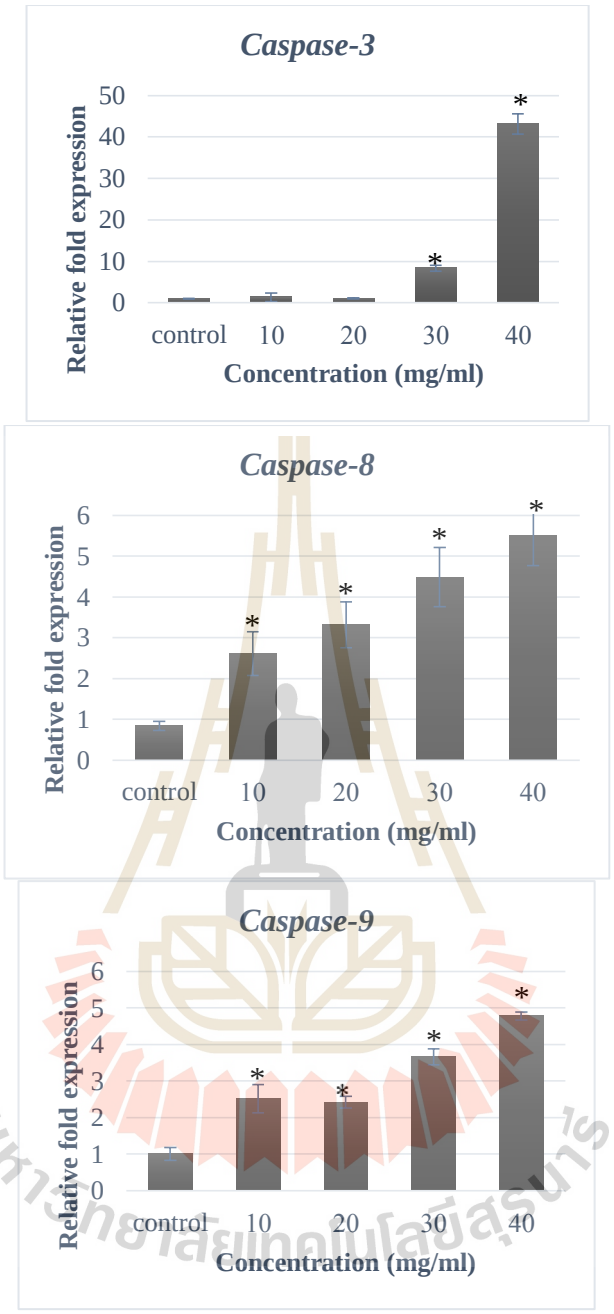

Figure 4.7 Secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptotic cells of human colorectal cancer cells. The mRNA expression level of apoptotic genes, *Caspase-3*, *Caspase-8* and *Caspase-9* were evaluated by semi-quantitative RT-PCR to present the relative expression of *Caspase-3*, *Caspase-8* and *Caspase-9* genes. Values are expressed as mean ± SD (n=3). *($p<0.05$) versus control cells.

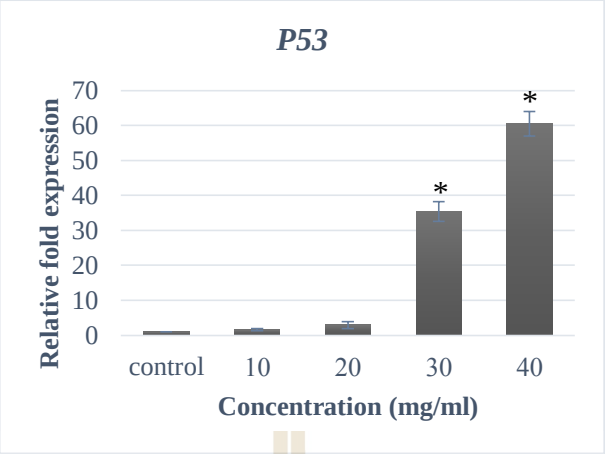

Figure 4.8 Secreted metabolites of *Lactobacillus* sp. 21C2-10 (SML) induced apoptotic cells of human colorectal cancer cells. The mRNA expression level of apoptotic genes, *P53* was evaluated by semi-quantitative RT-PCR to present the relative expression of *P53* gene. Values are expressed as mean ± SD (n=3).

*($p<0.05$) versus control cells.

4.4 Probiotic encapsulation

4.4.1 Physicochemical characteristics of microencapsulated probiotics

The chemical composition of the microencapsulated *Lactobacillus* sp. 21C2-10 including 33.671±2.062% protein, 15.844±0.201% fat, 9.517±0.285% crude fiber, and 0.211±0.032% ash on a wet basis. The % encapsulation yield (%EY) of *Lactobacillus* sp. 21C2-10 was showed at 92.558±0.504%. The cell densities of *Lactobacillus* sp. 21C2-10 were presented as 10.25±0.52 log CFU/g. The average diameter of the microencapsulated *Lactobacillus* sp. 21C2-10 was 88.581±15.020 µm. Optical microscope images showed spherical shapes. The sizes of the capsules can affect the texture of food as well as sensory quality (Rokka and Rantamaki, 2010).

Hansen et al. (2002) suggest that microcapsules with a size less than 100 μm could avoid a negative impact on the sensory attributes of food products.

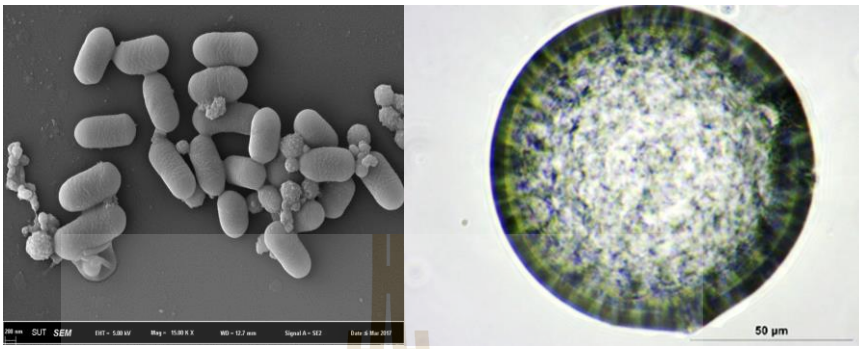

Figure 4.9 Morphological of *Lactobacillus* 21C2-10 and microencapsulated *Lactobacillus* sp. 21C2-10.

4.4.2 Survival of probiotic in free and microencapsulated forms after incubation in simulated gastric juices

With regard to its health effects, *Lactobacillus* sp. 21C2-10 should be resistant to the harsh environments of the gastric conditions. Therefore, the major objective of microencapsulation is to protect the probiotic bacteria from low pH. The pH of gastric juices is approximately 1.5-3.0 (Kos et al., 2000). The effect of microencapsulation by an emulsion technique on the survival of probiotic bacteria in free and microencapsulated forms during exposure to simulated gastric juices was evaluated. The results show that the survival rate (%) of *Lactobacillus* sp. 21C2-10 in microcapsules was significantly higher than the free probiotic bacteria during exposure to simulated gastric juices (Fig. 4.10). Microencapsulation of probiotic bacteria significantly ($P<0.05$) increased the survival of *Lactobacillus* sp. 21C2-10. *Lactobacillus* sp. 21C2-10 survived more than 10^9 CFU/g after 120 min of exposure to

gastric juices, suggesting that microencapsulated probiotic bacteria decreased less than one logarithm unit. At the end of the incubation time, the survival rate (%) of free cells decreased by 40.70±0.27%. On the other hand, microencapsulation of microcapsules showed significant ($P<0.05$) prevention during exposure times.

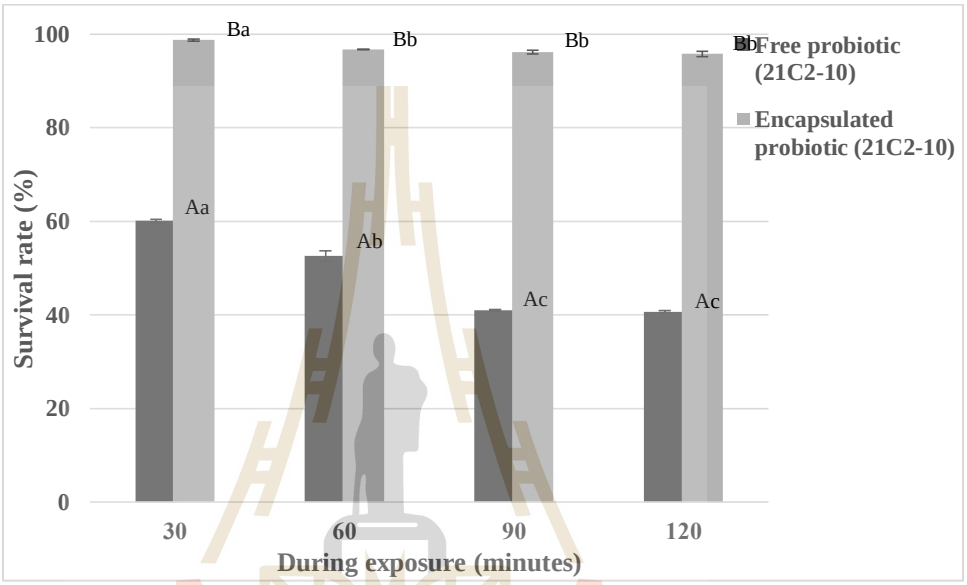

Figure 4.10 Survival rate (%) of *Lactobacillus* sp. 21C2-10 in free and encapsulated forms during exposure to SGJ for 120 minutes. Values represent means ± standard deviation (n = 3). A, B or other superscript capital letters denote significant differences among probiotic forms for the same incubation times. a,b or other superscript lower case letters denote significant differences among different incubation times for the same probiotic forms.

4.4.3 Survival of probiotic in free and microencapsulated forms after sequential incubation in simulated gastric and intestinal juices

The results for the survival rate (%) of *Lactobacillus* sp. 21C2-10 cells in free and microencapsulated froms after exposure to simulated gastrointestinal juices are shown in Fig. 4.11. Results showed that free probiotic cells were sensitive to SGJ. A significant ($P < 0.05$) reduction of 5.19±0.03 Log cycles was represented after exposure with SGJ for 60 min, resulting in a survival rate of 47.83±0.25%. It remained constant when exposure to SIJ for 240 min. Results show that microencapsulated probiotic bacteria can survive significantly ($P < 0.05$) higher than the free probiotic cells in simulated gastro-intestinal conditions. The survival rate of microencapsulated and free bacteria after exposure to SGJ for 60 min and sequential exposure with SIJ for 300 min was 87.87±1.27% and 39.67±0.15%, respectively. This study has shown that microencapsulation can prevent the loss of probiotic bacteria during exposure to gastrointestinal conditions. These results are similar to those of Nawong et al. (2017) which suggest that microencapsulated probiotics using maltrodextrin and gelatin as wall materials which have been cross-linked by transglutaminase significantly enhanced the viability of *Lactobacillus* spp. after exposure to exposure to SGJ for 60 min and sequential exposure with SIJ 180 min at 37^0C. Leach et al. (1987) suggested that the low pH in SGJ could increase H^+ concentration in extracellular space. Therefore, an external pH of close to 2.0 will inhibit enzymes in many kinds of bacteria. Furthermore, bacteria maintain their H+concentrations using energy (ATP) to drive protons by way of backward ATPase provided to bacterial cells to lose their energy (ATP) source, which cannot metabolize nutrients. Thus, bacteria begin to die. Moreover, Zhu et al. (2006) suggest that bile salt

can be toxic for probiotic bacteria and that many probiotic bacteria have bile salt hydrolases to reduce bile salt (Begley et al., 2006). Therefore, microencapsulation helps the survival of *Lactobacillus* sp. 21C2-10 after exposure to SGJ and sequential exposure to SGJ and SIJ. This study clearly shows that the wall materials prevent the contact of probiotic bacteria with the low pH environment, enzymes and bile salts due to the position of probiotic bacteria inside the microcapsules, the properties of gelatin, and resistance from pepsin by the actions of TGase enzymes that produced iso-peptide. These results show that microencapsulated probiotic bacteria can be appropriately added to food products.

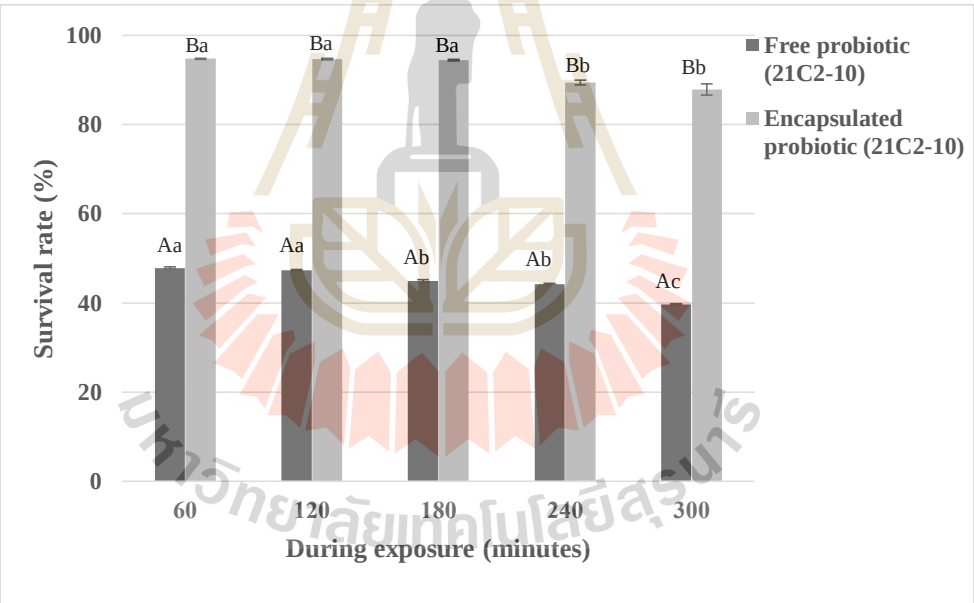

Figure 4.11 Survival rate (%) of *Lactobacillus* sp. 21C2-10 in free and microencapsulated forms during exposure to SGJ for 60 minutes and sequential exposure with SIJ for 240 minutes. Values are representatives of means ± standard deviation (n = 3). A, B or other superscript capital letters denote significant differences among

probiotic forms for the same incubation times. a,b or other superscript lower case letters denote significant differences among different incubation times for the same probiotic forms.

4.5 Ice cream probiotics

4.5.1 Physicochemical characteristics of ice cream

The chemical composition of ice cream samples are shown in Table 4.2. The results show that no significant differences ($p>0.05$) were observed in treatments T1, T2, and T3 for fat, ash, crude fiber, and moisture content. However, ice cream containing microencapsulated *Lactobacillus* sp. 21C2-10 had significantly ($p<0.05$) higher protein content than ice cream containing free *Lactobacillus* sp. 21C2-10 and ice cream with no added probiotic bacteria.

Table 4.2 Chemical composition of probiotic ice cream (%wet basis).

Chemical composition	Crude fat	Crude protein	Ash	Crude fiber	Total solid
T1	14.055 ±0.592[a]	6.519 ±0.504[a]	0.558 ±0.025[a]	0.054 ±0.018[a]	36.839 ±0.488[a]
T2	14.049 ±0.524[a]	6.561 ±0.501[a]	0.549 ±0.024[a]	0.049 ±0.014[a]	36.848 ±0.562[a]
T3	14.051 ±0.156[a]	6.837 ±0.369[b]	0.557 ±0.021[a]	0.052 ±0.221[a]	37.110 ±0.980[a]

Values are shown as means ± standard deviation (n = 30). Values followed by different superscripts in the same column are significantly ($p<0.05$) different.

4.5.2 Survival of probiotic in free and microencapsulated forms in ice cream during storage time at -20 ^{0}C for 180 days

Figure 4.12 shows the survival rate (%) of probiotic in free and microencapsulated in the ice cream samples during 180 days of storage at -20^{0}C. The survival rate (%) of the microencapsulated *Lactobacillus* sp. 21C2-10 in ice cream samples (93.858±0.358%) was significantly higher than the survival rate of free *Lactobacillus* sp. 21C2-10 in ice cream samples (72.024±0.409%). The survival rate (%) of microencapsulated *Lactobacillus* sp. 21C2-10 in ice cream samples was also significantly ($p<0.05$) higher than free probiotic bacteria during frozen storage. Therefore, the microencapsulation of probiotic bacteria in capsules can increase the viability of probiotic bacteria in ice cream samples. These results are similar to those of other authors. For example, Sheu and Marshall (1993) suggested that entrapped *Lactobacillus spp.* increased viable probiotic bacteria in ice cream. Homayouni et al. (2008) found that encapsulated probiotic bacteria could enhance the viable probiotic bacteria in synbiotic ice cream. Magarinos et al. (2007) also found that probiotic ice cream containing encapsulated acto acidophilus stored at -25^{0}C for 60 days of storage had a survival rate of 87%. Champagne et al. (2015) suggested that microencapsulated *Lactobacillus rhamnosus* had a viable count higher than free cells in ice cream during 210 days of storage. The reduction of probiotic bacteria in ice cream is possibly due to freezing injury, which causes the death of cells (Kailasapathy and Sultana, 2003), which may damage the cell walls (Cruz et al., 2010). Kailasapathy and Sultana (2003) reported that the survival of probiotic bacteria against unfavorable conditions such as oxygen toxicity, freezing, and storage at lower temperatures in ice cream. However, the neutral pH of ice cream can protect the probiotic bacteria in free forms

(Homayouni et al., 2008). This study suggests that damage to probiotic cells is likely to be caused by the formation of ice crystals and oxygen toxicity. However, microcapsules could protect cells from crystallized water, providing resistance to freezing damage and could protect cells from oxygen toxicity. Further, the number of *Lactobacillus* sp. 21C2-10 (8.358±0.214 log CFU/g) followed the recommended therapeutic minimum limit of 7 log CFU g-1 for up to 180 days of storage at -20^{0}C.

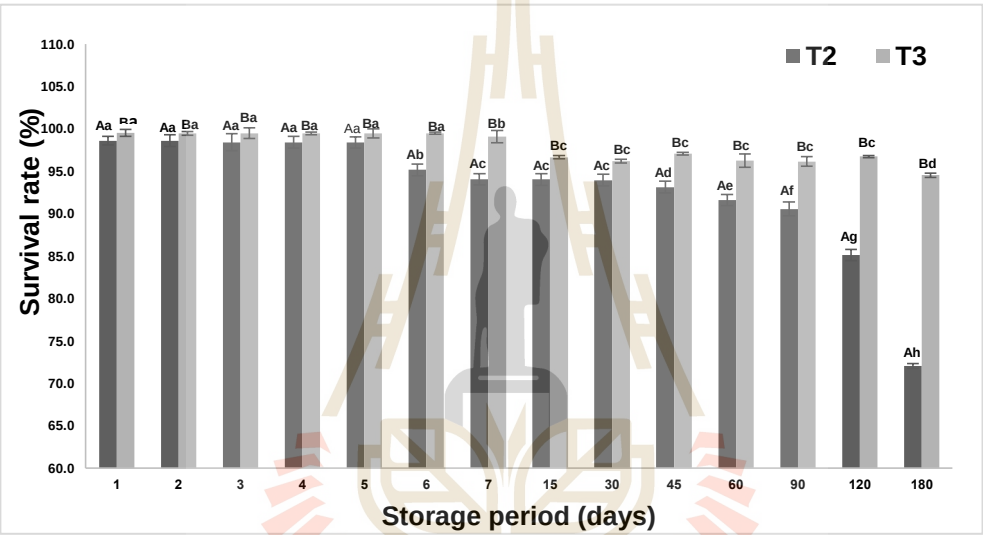

Figure 4.12 Survival rate (%) of ice cream containing free cells of *Lactobacillus* sp. 21C2-10 (T2) and ice cream containing microencapsulated cells of *Lactobacillus* sp. 21C2-10 (T3) during storage time stored at -20ºC. Values show means±standard deviation (n = 3). A,B or other superscript capital letters denote significant differences among ice cream formulations for the same number of days of storage. a,b or other superscript lower case letters denote significant differences for a different number of days of storage times.

4.5.3 pH and titratable acidity (%) values

The results of the pH and titratable acidity (%TA) values for different treatments of ice cream samples during storage time at -20^{0}C are presented in Figure 4.13. The pH and titratable acidity (%) of the ice cream samples are not significantly (P>0.05) different between T1 and T3 when evaluated between day 0 to day 180 (P>0.05). However, the pH value of ice cream containing free probiotic bacteria (T2) had the lowest value, which was significant (P<0.05) when compared to ice cream containing microencapsulated probiotic bacteria (T3) and ice cream with no added probiotic bacteria (T1). The titratable acidity (%) (as lactic acid) of ice cream containing free probiotic bacteria (T2) had the highest value when compared to ice cream containing microencapsulated probiotic bacteria (T3) and ice cream with no added probiotic bacteria (T1) during180 days of storage. This study shows that the ice cream containing free probiotic bacteria increased titratable acidity, while pH decreased due to activity of lactic acid bacteria during processing. However, ice cream containing microencapsulated probiotic bacteria showed minor changes. Kailasapathy and Sultana (2003) suggested that titratable acidity (%) increases after production could be due to the activity of probiotic cells. It has been shown that lactic acid bacteria may release enzymes which could convert lactose into lactic acid and increase acidity in dairy food products (Widyastuti and Febrisiantosa, 2014). This result shows that microencapsulated probiotic bacteria have more stability from the environment. However, non-significant changes in the pH and titratable acidity of ice cream occurred during storage time. Similar results were reported by Moeenf-ard and Tehrani (2008) found that the titratable acidity of frozen yoghurt continued constant during storage at -20^{0}C for 180 days. They attributed the constancy to the low storage temperature (-20^{0}C). Alamprese et al. (2002) suggested that acidity in ice

cream containning microencapsulated *Lactobacillus johnsonii* stored at -16 and -28^{0}C for 90 days remained unchanged. They suggested that the pH value and titratable acidity (%) of probiotic ice cream showed no significant difference during storage at -20^{0}C. The present study confirms that adding microencapsulated probiotic bacteria can decrease the activity of probiotic bacteria, providing no effect on the total lactic acid of ice cream after production. Furthermore, the results show that the acidity of ice cream is essentially constant during storage at freezing temperatures.

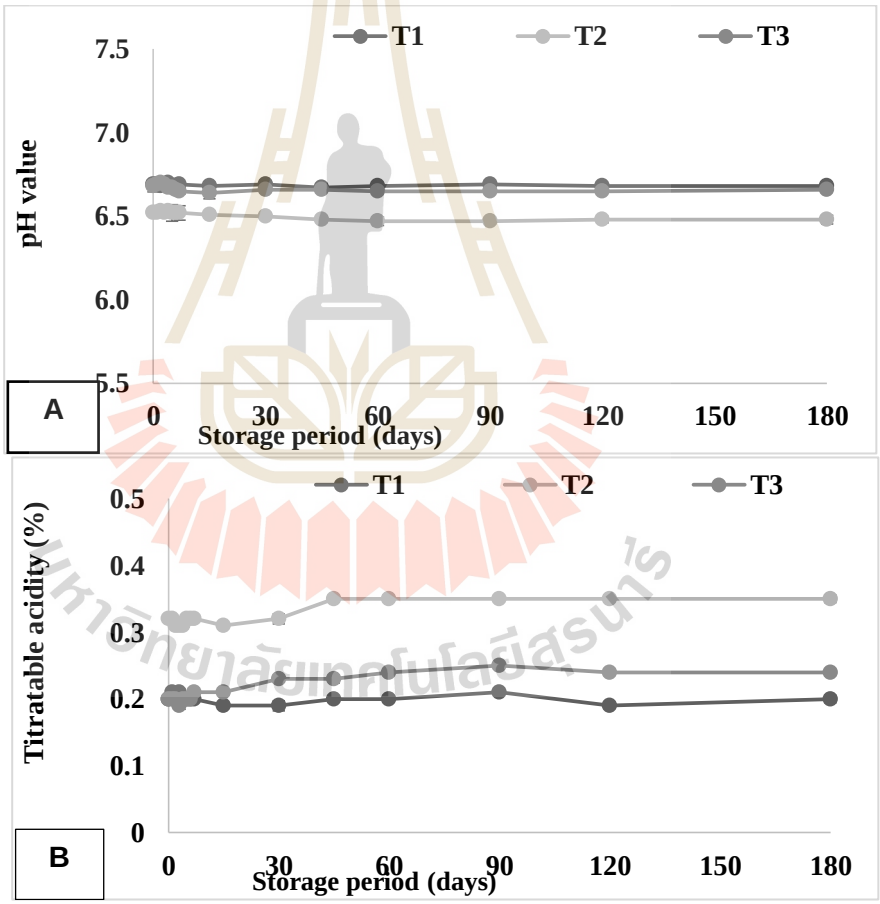

Figure 4.13 pH value (A) and titratable acidity (%) (B) of ice cream without *Lactobacillus* sp. 21C2-10 (T1), ice cream containing free cells of *Lactobacillus* sp. 21C2-10 (T2) and ice cream containing

microencapsulated cells of *Lactobacillus* sp. 21C2-10 (T3) during storage time stored at -20ºC. Values show means ± standard deviation (n = 3).

4.5.4 Microbial spoilage analysis

Total aerobic bacteria, Coliforms, *E. coli*, yeast and molds were not found in any ice cream treatment (T1, T2, T3) (data not shown).

4.5.5 Sensory evaluation

The sensory scores for ice cream samples after 1 and 180 days of storage using a 9-point hedonic scale for color and appearance, flavor and taste, body and texture and total acceptability are given in Table 4.3. The sensory scores for ice cream samples (T1 and T3) are not significantly ($P>0.05$) different after 1 and 180 days of storage time. Only the ice cream containing free probiotic bacteria (T2) showed significantly ($P<0.05$) lower scores for flavor, taste and total acceptability when compared to ice cream with no added probiotic bacteria (T1) and ice cream containing microencapsulated probiotic bacteria (T3) after 1 and 180 days of storage time. This result is similar to García-Ceja et al. (2015), who reported that set style yogurt containing free probiotic bacteria showed significantly lower scores for flavor and taste. In addition, when compared to set style yogurt containing microencapsulated probiotic bacteria the results showed significantly higher total titratable acidity (%) than the other ice cream samples (T1 and T3) due to ice cream containing free probiotic bacteria.

Table 4.3 Sensory properties of ice creams after 1 and 180 days of frozen storage.

Sensory attributes		During storage time	
		1 day	180 days
Color and appearance	T1	6.399±0.061$^{a, A}$	6.378±0.058$^{a, A}$
	T2	6.375±0.042$^{a, A}$	6.350±0.053$^{a, A}$
	T3	6.391±0.048$^{a, A}$	6.378±0.057$^{a, A}$
Body and texture	T1	6.752±0.026$^{a, A}$	6.754±0.023$^{a, A}$
	T2	6.752±0.034$^{a, A}$	6.739±0.037$^{a, A}$
	T3	6.739±0.039$^{a, A}$	6.752±0.036$^{a, A}$
Flavor and taste	T1	6.985±0.027$^{a, A}$	6.982±0.028$^{a, A}$
	T2	6.778±0.035$^{b, B}$	6.793±0.034$^{b, B}$
	T3	6.97±0.036$^{a, A}$	6.98±0.034$^{a, A}$
Total acceptability	T1	7.731±0.075$^{a, A}$	7.718±0.0277$^{a, A}$
	T2	7.684±0.034$^{b, B}$	7.673±0.038$^{b, B}$
	T3	7.720±0.032$^{a, A}$	7.708±0.034$^{a, A}$

Values show means ± standard deviation (n = 30). A, B or other superscript capital letters denote significant ($p<0.05$) differences among ice cream formulations for the same number of days of storage. a, b or other superscript lower case letters denote significant ($p<0.05$) differences for different numbers of days of storage times.

4.5.6 Survival of probiotic in free and microencapsulated froms in ice cream during sequential exposure to gastro-intestinal juices.

The survival of *Lactobacillus* sp. 21C2-10 during incubation times in simulated gastric juices (SGJ) (Figure 4.14A) and under sequential incubation times in simulated gastro-intestinal juices (Figure 4.14B) affected the amount of viable *Lactobacillus* sp. 21C2-10 in ice cream samples. The results show that the amount of viable *Lactobacillus* sp. 21C2-10 decreased when the incubation time was increased. Comparisons of the viability of *Lactobacillus* sp. 21C2-10 in ice cream containing free and microencapsulated cells after exposure to SGJ at 37^0C for 120 mins under anaerobic conditions was investigated. In this case, the survival rate (%) of ice cream containing microencapsulated *Lactobacillus* sp. 21C2-10 (95.892±0.198%) was significantly ($p<0.05$) higher than ice cream with free *Lactobacillus* sp. 21C2-10 (45.355±0.451%) when exposed to SGJ for 120 mins. Figure 4.14B shows the viability of *Lactobacillus* sp. 21C2-10 in ice cream after exposure to SGJ for 60 mins and SGJ for 240 mins at 37^0C under anaerobic conditions. The results show that the survival rate (%) of ice cream containing microencapsulated *Lactobacillus* sp. 21C2-10 (94.018±0.015%) is significantly ($p<0.05$) higher than ice cream containing free *Lactobacillus* sp. 21C2-10 (43.201±0.307%) when exposed to SGJ (60 mins) and SIJ (240 mins). In addition, Optical microscope images showed that microcapsule beginning disintegrated after 60 min in SGJ and sequential exposure to SIJ for 60 min (Figures 4.15C) and fully disintegrate after 60 min in SGJ and sequential exposure to SIJ for 120 min (Figures 4.15C) before reaching the large intestine. Moreover, Figure 4.16 shows scanning electron micrographs of *Lactobacillus* sp. 21C2-10 in ice cream samples when exposed to SGJ for 0 min (Figure 4.16A, D), SGJ for 60 mins (Figure

4.16B, E) and SGJ for 60 mins and sequential exposure to SIJ for 240 mins (Figures 4.16C, F). The SEM micrographs show morphological changes on the cell surface. Untreated ice cream shows normal morphological structure. For ice cream containing free *Lactobacillus* sp. 21C2-10 during sequential exposure to simulated gastro-intestinal conditions (Figure 4.16A-C), the SEM micrographs show a large amount of the probiotic bacteria had a number of wrinkled and shrunken structures on the cell surface, as shown by black arrows. In contrast, ice cream containing microencapsulated probiotics show changes on the cell surface at the end of the incubation time (Figures 4.16D-F). Some particle cells show shrunken surfaces. This study has shown that microencapsulation can significantly prevent probiotic bacteria during exposure to gastrointestinal conditions. This is similar to Ribeiro et al. (2014), who observed that yogurt containing microencapsulated *Lactobacillus acidophilus* showed more endurance to simulated gastro-intestinal juices than yogurt containing free *Lactobacillus* acidophilus. Further, Matias et al. (2016) reported that ice cream containing free *Lactobacillus acidophilus* LA-5 showed physiological change when stress was induced on the gastro-intestinal tract (in vitro assay). Moreover, Nawong et al. (2017) suggest that microencapsulated probiotic using maltrodextrin and gelatin as wall materials which has been cross-linked by transglutaminase significantly enhanced the viability of *Lactobacillus* spp. after exposure to SGJ and sequential exposure to SIJ for 240 mins at 37^0C. Leach et al. (1987) suggested that the low pH in SGJ could increase H+ concentration in extracellular space. Therefore, an external pH of close to 2.0 will inhibit enzymes in many kinds of bacteria. Furthermore, bacteria maintain their H+concentrations using energy (ATP) to actively eliminate protons by way of backward ATPase provided to bacterial cells to lose their energy (ATP)

source, which cannot metabolize nutrients. Thus, bacteria begin to die. Moreover, Zhu et al. (2006) suggest that bile salt can be toxic for probiotic bacteria and that many probiotic bacteria have bile salt hydrolases to reduce bile salt (Begley et al., 2006). In this study, it was observed that the majority of losses of cell viability occurred during exposure to SGJ for 60 mins. In contrast, few alters occurred during exposure to SIJ for 180 mins.

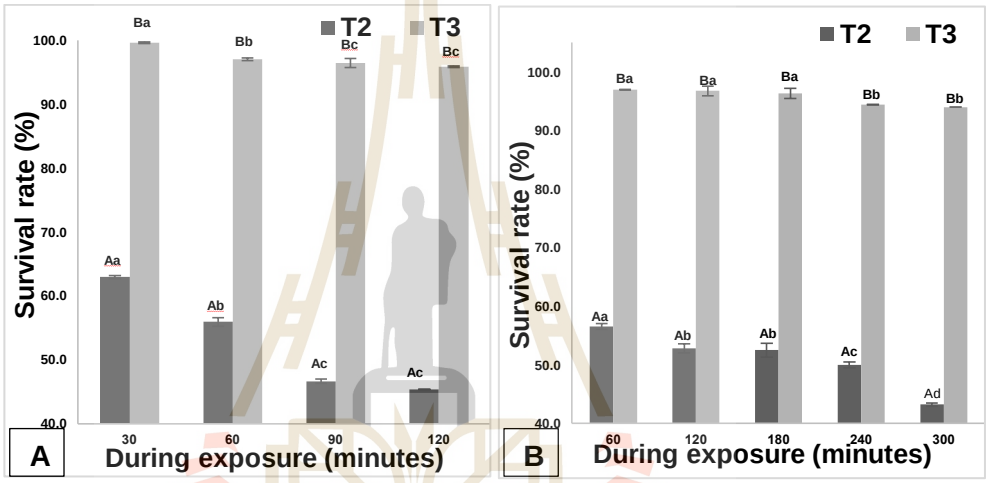

Figure 4.14 The survival rate (%) of *Lactobacillus* sp. 21C2-10 in ice cream after exposure to simulated gastric juices at 37°C for 120 minutes under anaerobic conditions (A) and the survival rate (%) of *Lactobacillus* sp. 21C2-10 in ice cream after sequential exposure to simulated gastric juices for 60 minutes and simulated intestinal juices for 240 minutes at 37ºC under anaerobic conditions (B). Values show means ± standard deviation (n = 3). A, B or other superscript capital letters denote significant differences among ice cream formulations. a, b or other superscript lower case letters denote significant differences for different incubation times for the same ice cream.

Therefore, microencapsulation helps the survival of *Lactobacillus* sp. 21C2-10 in ice cream after exposure to SGJ and sequential exposure to SGJ and SIJ. These results show that the total number of viable probiotic bacteria in the microcapsules (8.098±0.124 log CFU/g) remain within the values required by the International Dairy Federation.

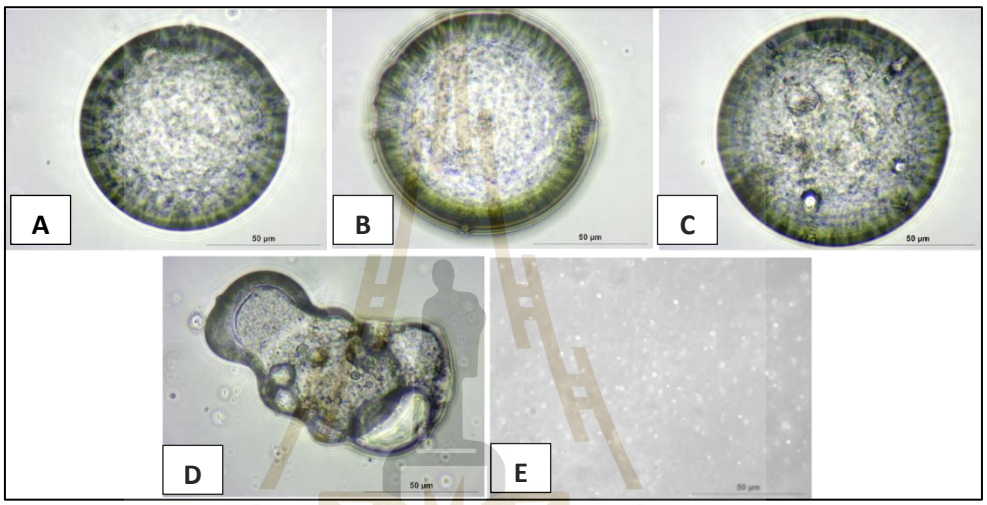

Figure 4.15 Morphology of microencapsulated probiotics in ice cream when exposed to gastro-intestinal juices show morphological changes of *Lactobacillus* sp. 21C2-10 in ice cream without exposure to simulated gastric juices (A), after exposure to simulated gastric juices for 60 minutes (B), after sequential exposure to simulated gastric juices for 60 minutes and simulated intestinal juices for 60 minutes (C), after sequential exposure to simulated gastric juices for 60 minutes and simulated intestinal juices for 120 minutes (D) and after sequential exposure to simulated gastric juices for 60 minutes and simulated intestinal juices for 240 minutes at 37ºC under anaerobic conditions (E).

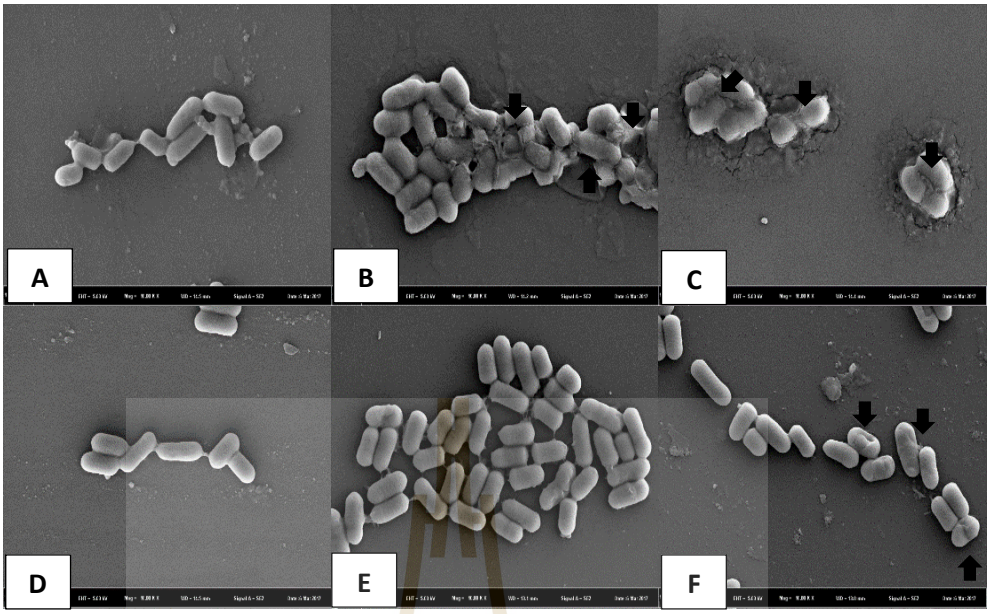

Figure 4. 16 Scanning electron micrographs showing morphological changes of cell surface of *Lactobacillus* sp. 21C2-10 in ice cream without exposure to simulated gastric juices (A, D), after exposure to the simulated gastric juices for 60 minutes at 37ºC under anaerobic conditions (B, E), after sequential exposure to simulated gastric juices for 60 minutes and simulated intestinal juices for 240 minutes at 37ºC under anaerobic conditions (C, F). Figs A-C above show ice cream containing free cells of *Lactobacillus* sp. 21C2-10 and Figs D-F above show ice cream containing microencapsulated cells of *Lactobacillus* sp. 21C2-10 ice cream containing microencapsulated cells of *Lactobacillus* sp. 21C2-10

CHAPTER V

CONCLUSIONS

Lactobacillus sp. 21C2-10 was isolated from cassava pulp from Nakhon Ratchasima province which showed strengthened cell adhesion to Caco-2 human colorectal adenocarcinoma cells of 14.44±0.58%. The secreted metabolites from *Lactobacillus* sp. 21C2-10 showed cytotoxic effects of Cac0-2 cells when evaluated using a MTT assay. The secreted metabolites from *Lactobacillus* sp. 21C2-10 caused a significant ($P < 0.05$) decrease in cell viability of Caco-2 (%), in a dose-dependent manner. The cell viability of Caco-2 cells was inhibited by secreted metabolites of *Lactobacillus* sp. 21C2-10 at 10, 20, 30 and 40 mg/ml of 88.29±5.62%, 64.33±2.67%, 52.59±5.63%, and 28.74±0.42%, respectively. This result shows that the anti-proliferative effect of SML on Caco-2 cancer cells had significant differences with control (untreated) and MRS-treated groups. Evaluation of secreted metabolites of *Lactobacillus* sp. 21C2-10 causing anti-proliferative effects were evaluated using a tail image base cytometer for assessing the induction of apoptosis on Caco-2 cancer cells. The results show that SML significantly increased apoptotic cells (%) compared with the control group (Fig. 2). The treated cells (40 mg/ml) showed live, dead, apoptotic cells (%) of 26.33±4.62%, 2.00±1.00% and 71.67±4.73%, respectively. The treated cell lines showed apoptotic cells in a dose-dependent manner. Moreover, the effects of SML on the morphological changes of Caco-2 cancer cells from DAPI staining method. The results show that the Caco-2 cells were treated with 10, 20, 30

and 40 mg/ml of SML. Caco-2 cells represent cell apoptosis, including membrane blebbing, cell shrinking and condensed chromatin and apoptotic bodies. In contrast, none of these apoptotic signals were represented in untreated Caco-2 cells. Furthermore, these results indicate that SML induced the apoptotic pathway in Caco-2 cells through the gene expression of BAX, P53, Caspase 3, 8 and 9 in Caco-2 cancer cell lines increased significantly with increased doses of SML. However, SML decreased the gene expression of Bcl-2 significantly when compared with the control. Moreover, the gene expression of pro-apoptotic genes were increased, while anti-apoptotic genes decreased. These results confirm that secreted metabolites from *Lactobacillus* sp. 21C2-10 indicated that apoptosis is the major pathway for cell death. *Lactobacillus* sp. 21C2-10 adhered to Caco-2 cells and anti-cancer properties by means of apotosis pathway, indicating probiotics can be appropriately added to food products.

Microencapsulation of *Lactobacillus* sp. 21C2-10 by an emulsion technique using maltrodextrin and gelatin as wall materials helped improve the survival of probiotics when exposed to harsh environments. Therefore, encapsulation will help protect probiotics until the site of action to adhesion and induction of apoptotic pathways. The microencapsulated *Lactobacillus* sp. 21C2-10 prepared using an emulsion technique showed the chemical composition included 43.108±2.062% protein, 20.274±0.201% fat, 12.182±0.285% crude fiber, and 0.921±0.032% ash on a dry basis. The % encapsulation yield (%EY) of *Lactobacillus* sp. 21C2-10 was observed at 92.558±0.504%. The cell densities of *Lactobacillus* sp. 21C2-10 were 10.25±0.52 log CFU g-1. The average diameter of microencapsulated *Lactobacillus* sp. 21C2-10 was 88.581±15.020 µm. Phase contrast microscope images showed

spherical shapes. These results show that microencapsulated probiotic bacteria can survive significantly ($p < 0.05$) longer than free probiotic cells in simulated gastro-intestinal conditions. The survival rate of probiotic in microencapsulated and free forms after exposure to SGJ and sequential exposure to SIJ for 300 min was 87.87±1.27% and 39.67±0.15%, respectively. This study has shown that microencapsulation can significantly prevent probiotic cells during exposure to gastrointestinal juices. When microencapsulated *Lactobacillus* sp. 21C2-10 is added to ice cream that no significant differences ($P>0.05$) were observed in fat, ash, crude fiber, and moisture content compared with ice cream to which no probiotics were added. However, ice cream containing microencapsulated *Lactobacillus* sp. 21C2-10 had significantly ($p<0.05$) higher protein content than ice cream containing free Lactobacillus sp. 21C2-10. The survival rate (%) of *Lactobacillus* sp. 21C2-10 after exposure to simulated gastro-intestinal conditions of ice cream containing microencapsulated cells and free cells of *Lactobacillus* sp. 21C2-10 was evaluated after 180 days of frozen storage. Sensory evaluation of the ice cream was conducted after 1 and 180 days of frozen storage. Ice cream containing microencapsulated cells showed a significantly ($p<0.05$) higher survival rate, lower acidity, and higher pH values compared to ice cream containing free cells after storage for 180 days. The addition of microencapsulated cells had no significant ($p>0.05$) effect on the sensory properties of the ice cream. After exposure to simulated gastro-intestinal juices for 5 hrs, the ice cream containing microencapsulated cells showed a significantly ($p<0.05$) higher survival rate compared to ice cream containing free cells. Therefore, microencapsulation of *Lactobacillus* sp. 21C2-10 by the emulsion technique using maltrodextrin and gelatin as wall material did not affect the sensory properties in the

ice cream samples and effectively protected probiotic bacteria during frozen storage and delivery into the human gastrointestinal tract. Moreover, the number of probiotic bacteria in the ice cream sample was between 8.358±0.214 log CFU/g at the end of the storage time. This amount of viable probiotic bacteria number is higher than that recommended by the International Dairy Federation (7 log CFU/g). Thus, it may protect probiotics until they adher to epithelial cells and prevent cancer by induction of apoptosis pathway. Furthermore, it may be possible to use various substrates such as soluble and insoluble fibers for fermentation by *Lactobacillus* sp. 21C2-10 in order to test anti-cancer properties in future research.

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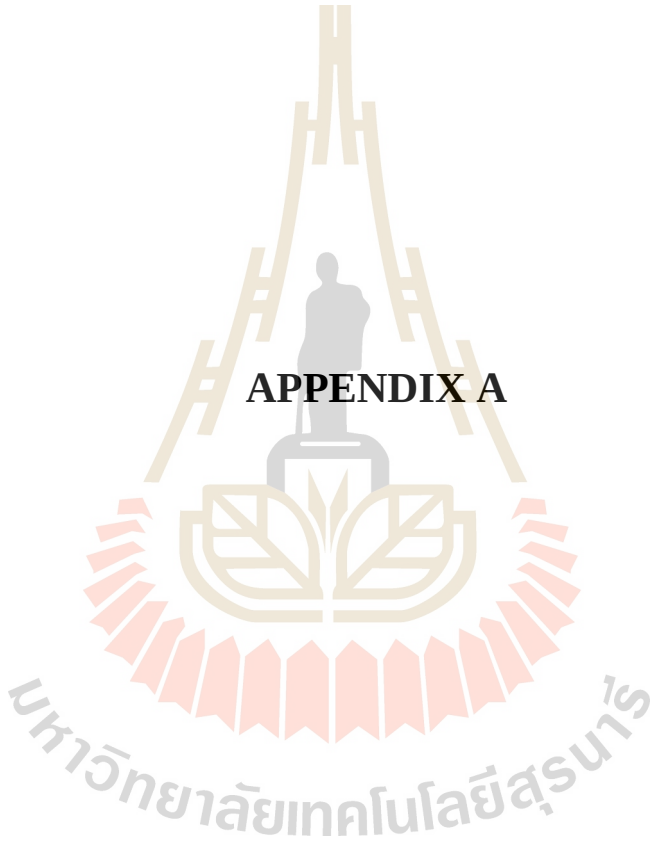
APPENDICES

APPENDIX A



1. MRS agar

MRS agar powder	67.15	g
Distilled water	1000.0	ml

2. Complete medium

DMEM	40.0	ml
FBS	4.5	ml
Non-essential amino acid	500	µl
L-glutamine	500	µl
Penicillin-streptomycin	500	µl

3. 50X Phosphate buffered saline (50X PBS)

Tris-base	121	g
Glacial acetic	28.5	ml
0.5 M EDTA pH 8.0	9.25	g
Distilled water	500.0	ml

5. MTT solution (0.5 mg/ml)

MTT	125.0	mg
Add PBS to bring volume up to	25.0	ml

6. Paraformaldehyde (4%)

Paraformaldehyde	4	g
Add PBS to bring volume up to	100.0	ml

7. MRS broth

MRS broth powder	55.15	g
Distilled water	1000.0	ml

8. Gelatin (24% w/w)

Gelatin	24	g
Add Nacl (0.5% w/v) to bring volume up to	100.0	ml

9. Maltrodextrin (24% w/w)

Maltrodextrin	24	g
Add Nacl (0.5% w/v) to bring volume up to	100.0	ml

10. NaCl (0.5% w/v)

NaCl	0.5	g
Add distilled water to bring volume up to	100.0	ml

11. Span 85 (0.5% w/w)

Span 85	0.5	g
Add Nacl (0.5% w/v) to bring volume up to	100.0	ml

12. NaCl (0.85% w/v)

NaCl	0.85	g
Add distilled water to bring volume up to	100.0	ml

13. Tween 80 (0.5% w/v)

Tween 80	0.5	g
Add Nacl (0.5% w/v) to bring volume up to	100.0	ml

14. NaOH (40% w/v)

NaOH	40	g
Add Distilled water to bring volume up to	100.0	ml

15. Boric acid (4% w/v)

Boric acid	4	g
Add Distilled water to bring volume up to	100.0	ml

17. HCl (100 mM)

Hydrochloric acid (37% w/w)	833	µl
Add Distilled water to bring volume up to	100.0	ml

18. Sulfuric acid (1.25%)

Sulfuric acid	1.25	ml
Add Distilled water to bring volume up to	100.0	ml

19. NaOH (1.25%)

NaOH	1.25	g
Add Distilled water to bring volume up to	100.0	ml

20. Glutaraldehyde (2% w/v)

Glutaraldehyde	2	g
Add Distilled water to bring volume up to	100.0	ml

21. NaCl (0.2% w/w)

NaCl	0.2	g
Add Distilled water to bring volume up to	100.0	ml

22. $CaCl_2$ (0.5 mM)

$CaCl_2$	0.006	g
Add Distilled water to bring volume up to	100.0	ml

23. NaOH (0.01 M)

NaOH	0.04	g
Add Distilled water to bring volume up to	100.0	ml

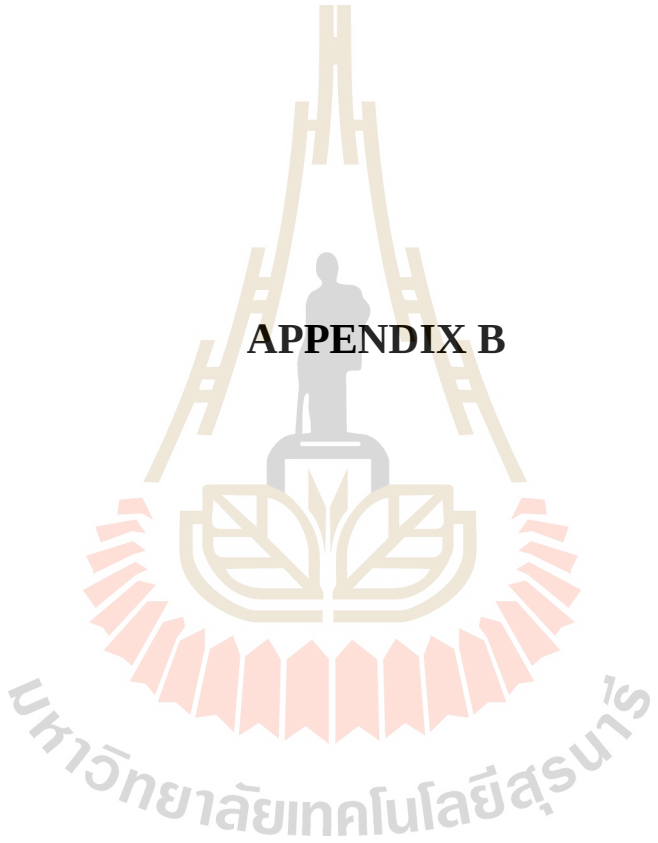
24. Phenolphthalein indicator (0.5% W/V)

Phenolphthalein	0.5	g
Add 95% ethanol to bring volume up to	100.0	ml

25. Potato dextrose agar (PDA)

PDA agar powder	39.0	g
Distilled water	1000.0	ml

APPENDIX B



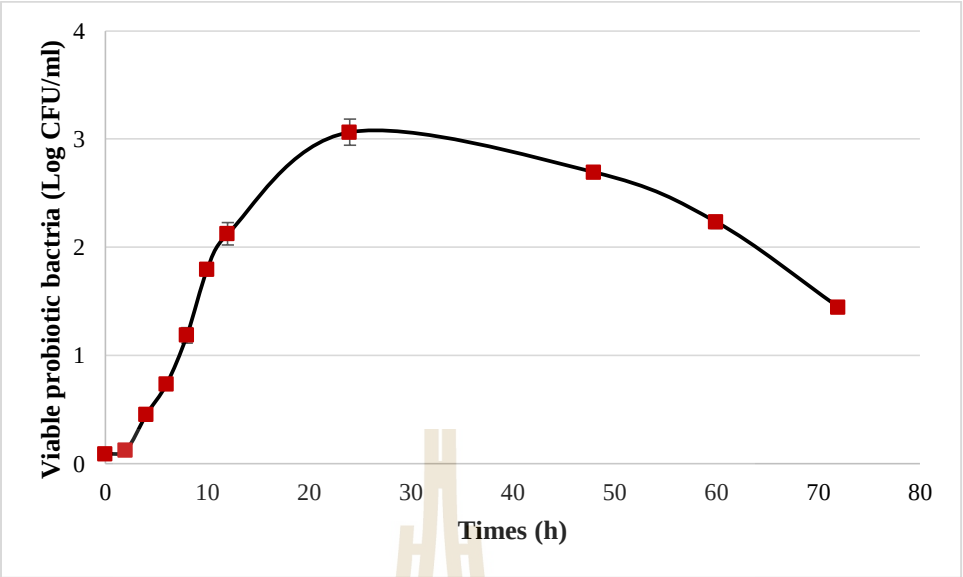


Figure 1 Growth of *Lactobacillus* sp. 21C2-10 on MRS broth at 37ºC.

มหาวิทยาลัยเทคโนโลยีสุรนารี

BIOGRAPHY

Ms. Sadudee Sengsaengthong was born in December 5, 1992 in Trad Province, Thailand. She received Bachelor's Degree in B.Sc. (Food Technology) from School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology in 2015. In 2015, she attended Master degree program at School of Food Technology, Suranaree University of Technology, Thailand. During her graduate study, she obtained opportunities to present her research work at the International Food Research Conference (UPM, Malaysia, 25-27th July, 2017).