FUNCTIONAL CHARACTERISTIC OF PROBIOTIC LACTIC ACID BACTERIA WITH BILE SALT HYDROLASE ACTIVITIES AND DEVELOPMENT OF PROBIOTIC PRODUCTS BY MICROENCAPSULATION

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NARATHIP PUTTARAT

A Dissertation Submitted in Partial Fulfillment of the Requirements
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BY

NARATHIP PUTTARAT

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The cholesterol-lowering activity of probiotic lactic acid bacteria (LAB) can ameliorate the effects of hypercholesterolemia. Thus, the purposes of this study were to isolate probiotic LAB with the ability to produce bile salt hydrolase (BSH) and cholesterol assimilation to be developed as probiotic products via microencapsulation using whey protein isolate (WPI) and nano-crystalline starch (NCS). In this study, *Lactobacillus reuteri* TF-7, *Enterococcus faecium* TF-18, and *Bifidobacterium animalis* subsp. *lactis* TA-1 expressed the most robust cholesterol-lowering activity and functional characteristics of probiotics. In the *in vivo* study, the oral administration of three strains contributed to the reduction of lipid levels and the modulation of gut microbiota. After developing probiotic products by microencapsulation, WPI-NCS microcapsules of probiotics could enhance survivability under gastrointestinal conditions and stability during long-term storage. The findings suggested that these probiotic products could be applied as an alternative treatment for metabolic disorders caused by hypercholesterolemia.

Keyword : Probiotics, Lactic acid bacteria, Bile salt hydrolase, Microencapsulation

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INTRODUCTION

The human body constitutes 90% of the microbial cells when compared with all human cells. The microorganisms living in the gastrointestinal tract are the most abundant and diversity which associated with host's health. These microorganisms can divide into two categories include good bacteria; probiotics and bad bacteria; opportunistic pathogens. Probiotics are defined by Food and Agriculture Organization of the United Nations/ World Health Organization (FAO/WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Consequently, probiotics are beneficial microorganisms that improve several health mechanisms and safe for the host protocooperation according to Generally Recognized As Safe guidelines (GRAS).(1) Moreover, probiotics can be found in various parts of the body, especially in the gastrointestinal microbial ecosystem. The important source of probiotics is functional food, as it can support metabolism to survive of probiotics. Functional foods can classify into two groups, including dairy based-foods, such as yogurt, curd, cheese, etc. While non-dairy based-foods, such as fruits, vegetables, breads, sausages, etc.(2) Currently, probiotics are being developed in various forms of supplement products to suit with the lifestyle. Synbiotics are a kind of probiotics product that develop with combination of prebiotics and probiotics in order to enhance probiotic viability, as prebiotics are a non-digestible carbohydrate molecule found in fruits and vegetables. They are able to promote probiotics growth, because probiotics can digest prebiotics to utilize for their metabolism.(3)

Presently, people more realize to health improvement because Thailand is entering the elderly society. Most important disease in the medical profession is non-communicable diseases (NCDs) which caused from hyperlipidemia, especially triglyceride and cholesterol.(4) Cholesterol is an essential biochemical molecule, all animal cells require cholesterol because it is a component of cell membrane to balance fluidity following fluid mosaic model. Moreover, cholesterol also acts as a precursor of vitamin D, bile acid, and some hormones.(5) Source of cholesterol consists of endogenous source which it is synthesized in the liver through mevalonate pathway to convert excess acetyl CoA to cholesterol with 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) as rate-limiting enzyme, while exogenous source obtains from absorption of cholesterol in foods which incorporates in the micelle form, following by conversion of cholesterol in micelle to cholesteryl esters with acyl-CoA cholesterol acyl transferase (ACAT), it is combined with apolipoprotein B-48 (chylomicron; ApoB-48) for formation of chylomicron to transfer into the liver, muscle as well as adipose tissues. In the event that people consume high cholesterol or fat diet for a long time, cholesterol is accumulated in extra-hepatic tissues.(6) It may contribute to hypercholesterolemia and related diseases. There are various alternative treatments and protections for hypercholesterolemia.(7) Previously, medical treatment and prevention with chemical drugs was commonly used in medical care for patient, but also there are several evidences demonstrated side effect from drugs administration. Currently, there are various natural products have been published their potential to reduce cholesterol such as herbal extract, soluble fiber, food supplement, and probiotic products, respectively. For probiotic products, previous researches indicated the effect of probiotics on hypercholesterolemia and related diseases that people who often administered probiotic products, they improved health status, as probiotics possess a functional properties to reduce cholesterol via several mechanisms. Cholesterol assimilation is a direct mechanism of probiotics to utilize cholesterol and some lipids for creation their cell membrane and biomolecules.(8) Additionally, some probiotic strains can produce bile salt hydrolase (BSH) enzyme to convert conjugated bile salt to deconjugated bile salt, which leading to the defective lipid emulsification and contribute to the reduction of lipid absorption in the intestine. Consequences of bile salt biodegradation by BSH still affect to stimulate cholesterol consumption in liver to create new bile salt which contribute to reduction of cholesterol in the body.(9) Therefore, probiotics in the gut microbiota is intrinsic factor to indicate health status.(10) Additionally, probiotics should safe for use and express several health promoting potential when administered into the body.

Hemolytic activity and antibiotic susceptibility are representative evaluation for safety, while antimicrobial activity, acid bile tolerance and adhesion are the essential properties of probiotics in the gut microbiota. Therefore, most probiotic products available on the market are developed with many probiotic strains to synergize health promoting potential of probiotic products. However, there are many factors in manufacturing process that directly affect to viability and stability (shelf life) of probiotics during production, storage, and distribution of products. These factors are temperature, moisture, acidity, osmotic pressure as well as oxygen content. The environmental factors in the gastrointestinal tract also affect to survival and growth of probiotics in the body after administration. These factors are hydrochloric acid in the stomach, detrimental bile salt as well as the gastrointestinal enzymes.(11) The toxicity of hydrochloric acid on probiotics is denaturation of protein in their cells, while bile salt toxicity can degrade phospholipid on cell membranes because it is amphipathic molecules.(12) These factor are the defense mechanism of host to inhibit invasion of microorganism. As mentioned above, these factors influence to viable cells of probiotics in products less than 10^6 colony forming unit (CFU)/gram until consumption, which is contradictory with Food and Drug Administration (FDA) provision that living probiotic cells in the product are not less than 10^6 CFU/gram throughout the shelf life.(13)

With such problems, the novel innovation and biotechnology are microencapsulation which has been applied in the probiotic supplement industry to enhance the viability and stability of probiotics. Microencapsulation is a method of probiotic sealing with the clinically proved biomaterials.(14) Consequently, probiotic cells are entrapped in a physical barrier to protect against external conditions or surrounding environment during processing, storage, and gastrointestinal passage. Encapsulated cells are able to release when they reach suitable environment in the intestine consists of anaerobic conditions, pH as well as temperature.(15) Additionally, probiotic cells are encapsulated with single or combination of biomaterials linked by covalent bond, which depend on the selection of the principle of encapsulation and suitable biomaterials. The common biomaterials are often used for microencapsulation, including whey protein, alginate, maltodextrin, starch, synthetic polymers, and miscellaneous compounds. Whey protein, alginate, and starch are commonly used in microencapsulation of probiotics, because they are cheap, easily prepare, and release entrapped cells from microcapsules in the intestine.(16) The techniques of microencapsulation are divided into two categories, including gelation technique to produce hydrocolloid beads or microgels and drying technique in order to produce dried powder microcapsule.(17)

Since probiotic products are alternative health care because their functional properties facilitate to health promoting effect, and also cholesterol-lowering mechanism by BSH activity is interested in order to develop as cholesterol-lowering probiotic products. However, viability and stability of probiotics in a product rapidly reduced in a time-dependent manner. Microencapsulation is a biotechnological process, which is used to develop for protection of probiotic cells from the various stress conditions. As described above, the aims of this study were to isolate three genera of probiotic lactic acid bacteria (LAB), including *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. with ability to express BSH activity, cholesterol assimilation, and functional properties. These genera of cholesterol-lowering probiotic LAB were developed for probiotic products via spray drying microencapsulation to enhance survivability and stability during passage through the gastrointestinal tract and long term storage of probiotic products, respectively.

Objectives

1. To isolate and select three genera of probiotic LAB with ability to produce BSH and assimilate cholesterol.

2. To characterize functional properties of cholesterol-lowering probiotic LAB *in vitro*.

3. To study the effect of cholesterol-lowering probiotic LAB in rat model.

4. To develop cholesterol-lowering probiotic products via spray drying microencapsulation.

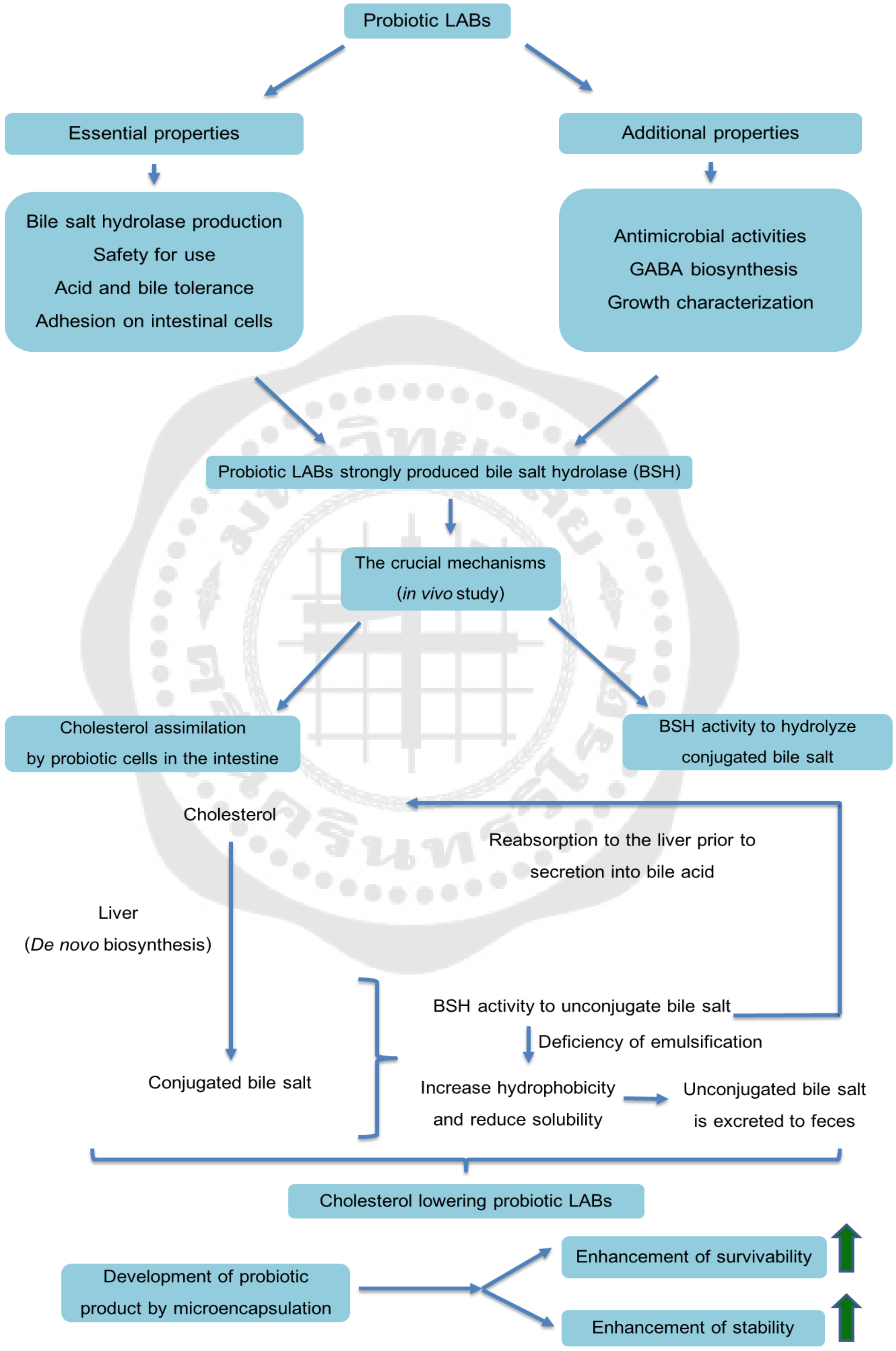
5. To study the survivability of cholesterol-lowering probiotic LAB in microcapsule products under the simulated gastrointestinal conditions.

6. To study the stability of cholesterol-lowering probiotic LAB during long term storage of microcapsule products.

Hypothesis

Microencapsulation of cholesterol-lowering probiotic LAB with ability to express BSH activity, cholesterol assimilation, and functional properties on health benefits, including *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. is able to increase survivability under the simulated gastrointestinal conditions and stability during long term storage.

Conceptual framework



CHAPTER 2

LITERATURE REVIEW

1 Human microbiota

The human body constitutes only 10% of human cells, while the other 90% are the cells of microorganisms that live within the body which known as human microbiota. Bacteria are the most microorganism that commonly found in the gastrointestinal tract, upper respiratory tract, lower urinary tract, and the integumentary system. The amount of bacteria in the body plays an important role in the physiological balance.(18) Moreover, there are evidences that microorganisms associated with health status and several pathological effects. Gut microbiota are the most importance to indicate the health status, because it can modulate nutrient homeostasis which contribute to promoting health status. Additionally, gut microbiota contain beneficial bacteria, which known as probiotics.(19) The variety of bacteria in the gastrointestinal tract is different as individual profile, which depend on genetics, gender, age, ethnicity, immune system, living behavior, stress, food consumption, and the surrounding environment.(20) These are factors that influence the amount and diversity of gut microbiota. The scientific evidence indicated that bacteria in the gastrointestinal tract play a role in various health aspects, especially probiotics.(21)

2 Definition of probiotics

The term of probiotic originally mentioned to microorganisms that can produce some substance to stimulate growth of other microorganisms.(22) In 1908, a Russian scientist named Élie Metchnikoff from the Louis Pasteur Institute observed that the Bulgarian people were a group of people who were long lived, when studied lifestyle and traditional foods. It was found that they consumed fermented milk on a daily basis, which possessed health benefits.(23) Additionally, the scientist found that lactobacilli could utilize sugar and convert to lactic acid in the fermented milk. After 1980, the conception of probiotic was called a group of microorganisms associated with beneficial effects in gastrointestinal microbalance of human and animal by Richard Buckminster

Fuller.(24) Additionally, the other scientists described probiotics as live microorganism that consumes in certain amount it can exhibit the health benefit. Until 2002, Food and Agriculture Organization of the United Nations (FAO) World Health Organization (WHO) had defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.(1) There is a trend about the benefits of probiotics that contribute to better health. Moreover, probiotics have been conducted involving in medical researches to clinical trials for alternative prevention and treatment in the future, especially metabolic disorders. Presently, there are several researches have proven various microorganisms to claim as probiotics. Consequently, several bacterial and fungal genera are listed as probiotics, including lactobacilli, bifidobacteria, enterococci, streptococci, and also *Saccharomyces* spp. These probiotics are commonly found in the environment which it is a resident microorganism in the human and animal as shown in Figure 1. (25) It is called human microbiota which exert essential role to promote health effect. Gut microbiota is the most diversity of microbial community, gastrointestinal environment suits to promote bacterial growth, because it is the source of nutrient metabolism. Therefore, probiotics play a crucial role in modulating nutrient metabolism of host and improve gut function. Furthermore, the physiological system involved with gastrointestinal tract can be maintain with functional properties of probiotics, including enteric nervous system, immune system, and excretory system. Oral route administration is suitable to replenish probiotics in the gut microbiota. The important source of probiotics is food products, including dairy based-food products such as milk, yoghurt, cheese, etc.(26) Non-dairy based-food products that found commonly are bread, fermented foods, sausages, and vegetables. (27) These food products can be found in traditional food of Thailand.

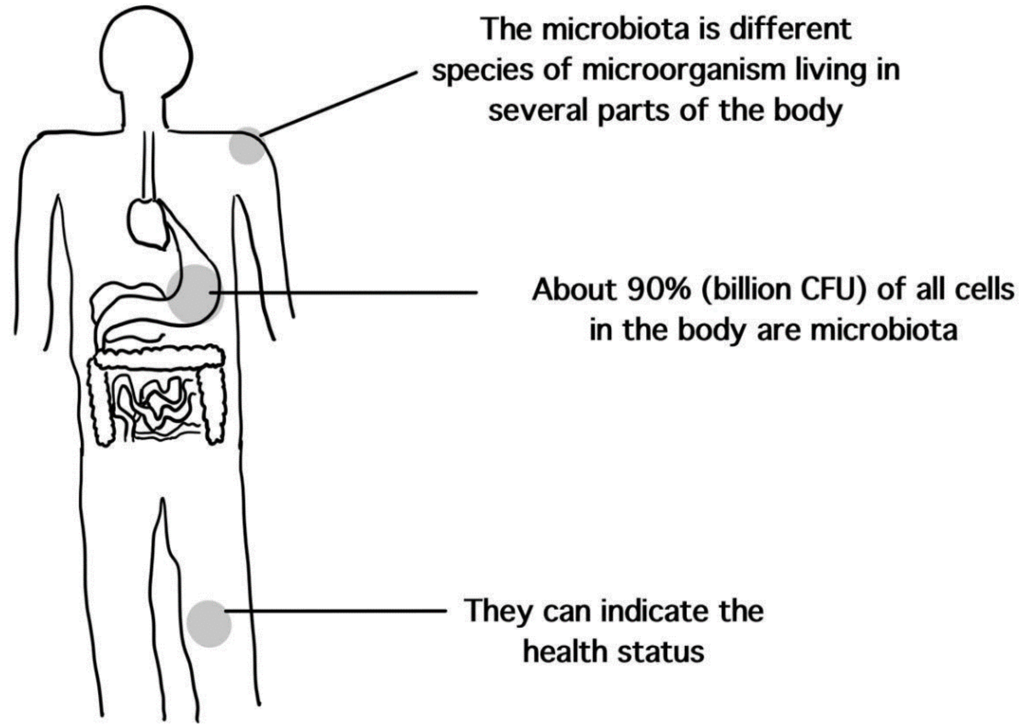


Figure 1 Microbiota and probiotics in the human body

2.1 Probiotic lactic acid bacteria

Most of probiotics are the member of lactic acid bacteria (LAB) which able to produce lactic acid by sugar fermentation. Lactic acid is a short chain fatty acid that contributes to balance fluid surrounding in the intestinal lumen.(28) Probiotic LAB are a group found in the gastrointestinal tract of human and animal origins as well as fermented food products. They are facultative anaerobe, gram-positive, catalase negative, non-spore forming, and non-motile bacteria. The most common probiotic LAB includes lactobacilli, bifidobacteria, and enterococci which have many species.(29)

2.1.1 Bifidobacteria

Bifidobacteria are a gram-positive bacilli, curve rod or di-branching (Y-shape), non-catalase producing, non-spore forming, non-motile, and anaerobic respiration. Several species have been isolated from human origins, animal origins, dairy, and fermented food products.(30) They are found less than other genera, as it is high sensitive to oxygen content in the atmosphere. The species of bifidobacteria that are reported to safe and can be used in commercial food products for human and animal include *Bifidobacterium bifidum*, *B. lactis*, *B. breve*, *B. infantis, B. animalis,* and *B. longum*.(31)

2.1.2 Lactobacilli

Lactobacilli are a major genera of probiotic LAB that is gram-positive bacilli, non-catalase producing, non-spore forming, non-motile and facultative or anaerobic respiration. Some species can grow at 30-40 ˚C which highly utilize sugar to produce lactic acid. They can be commonly found in human and animal origins because they constitute a significant component of the microbiota in the gastrointestinal tract and genital system. Lactic acid can protect host against potential damage from several pathogens.(32) Moreover, they are the most common probiotic LAB found in food, especially the dairy food product. Therefore, they are used in many food industries, species of Lactobacilli that are listed to safe and can be used in commercial food products for human and animal include *Lactobacillus reuteri, L. acidophilus, L. delbrueckii* ssp. *bulgalicus, L. paracasei, L. casei, L. salivarius, L. plantarum, L. amylovolus, L. crispatus, L. jhonsonii,* and *L. fermentum*.(33)

2.1.3 Enterococci

Enterococci are the gram-positive cocci that show in short chain morphology, non-catalase producing, non-spore forming, non-motile and facultative respiration. They are tolerant in environmental condition at 15-47 °C and pH of 4-8. However, they show gamma-hemolysis generally.(34) There are only two species which listed as probiotic LAB to use in commercial food products for human and animal, including *Enterococcus durans* and *E. faecium*.(35) Probiotics play an important role to improve digestive health, reduce the risk factor of disease, so they had been used to produce several supplemented products or functional foods such as yoghurt, kifir, sauerkraut, tempeh fermented soy bean, kimchi, miso, kombucha green tea, pickles traditional buttermilk, natto, some types of cheese, gum, probiotic powder, fermented food products, etc. There are many probiotics use for commercial food products according to show in Table 1.(36)

Table 1 The commercially used of lactic acid bacteria as probiotics

Probiotic strains	Products
Lactobacillus reuteri SD2112	Biogaia
Lactobacillus acidophilus LA1/LA5	Chr. Hensen
Lactobacillus delbrueckii ssp. *Bulgalicus* Lb12	
Lactobacillus paracasei CRL431	
Bifidobacterium animalis ssp *lactis* Bb12	
Lactobacillus acidophilus R0052	Institute Rosell
Lactobacillus rhamnosus R0011	
Lactobacillus acidophilus LB	Lacteol Laboratory
Lactobacillus paracasei F19	Medipharm
Bifidobacterium longum BB536	Morinaga Milk Industry Co. Ltd.
Lactobacillus jhonsonii La1	Nestle
Lactobacillus casei Shirota	Yakult

2.2 Criteria of probiotics

The microorganism which shows the mechanism of beneficial health effects in term of “PAFPM” from protection, absorption, function, production, and modulation that are general probiotic properties according to summarize in Table 2 include: the microorganisms are listed as probiotics should be generally recognized as safe (GRAS)(37), specific species of human origins are most suitable, the most probiotics localize in intestine so they should be tolerant to the gastrointestinal condition of hydrochloric acid and bile salt, produce antimicrobial substances, establish in the intestine, inhibit pathogen colonization and also survive during passage through the industrial processes and incorporation in food production.(38)

Table 2 Criteria of probiotic selection

Probiotic strains
Generally recognized as safe (GRAS) status
Specific strains of human origin
Non-pathogenic bacteria
Resistance to the gastrointestinal condition
Adherence to the gastrointestinal epithelial cells
Establishment to the gastrointestinal tract
Production of antimicrobial substances
Ability to survive during passage through the industrial technology and incorporation in food production

Moreover, some probiotic strain can express specific proficient of health beneficial effects. There are many previous studies discovered the crucial mechanism of specific properties, including resistance to enteric pathogen via production of antimicrobial peptide and lactic acid,(39) probiotic-mediated immunomodulation with diverse immune cells, detoxification, and excretion of toxic microbial metabolites because some probiotics localized in the intestine can produce metabolic enzyme to neutralize microbial toxin in the gastrointestinal tract, anti-hyperlipidemia activity via production of bile salt hydrolase (BSH) enzyme and assimilation of cholesterol to create their cell membrane, and prevention of urogenital Infection because probiotics adhere on epithelial cells in the vagina and urinary tract, which lead to competitive exclusion of pathogen.(40)

2.3 Probiotic proficiency on the beneficial health effects

Probiotics exert highly beneficial health effect when administer in suitable amount. Therefore, consumption of foods containing probiotics can promote health and prevent the pathogenesis of several disorders. There are many health benefits of probiotics as describe below.(41)

2.3.1 Anti-inflammation of gastrointestinal tract

The colonization of probiotics on the intestinal mucosa can prevent gastrointestinal inflammation, as their antimicrobial peptide substances, including bacteriocin, defencin, and cathelicidin can exhibit growth of pathogen by formation of pore on the cell membrane which lead to cell lysis. The previous study had been reported that administration of probiotic supplement with antimicrobial peptide of lactobacilli could reduce severity of gastrointestinal inflammation in the immunocompromised patients, which contribute to dysbiosis prevention resulting from modulation of the intestinal microbiota.(42)

2.3.2 Probiotic-mediated immunomodulation

Host's immunity is a coordinating system of biological molecules and organs to defend foreign substances. The immune system can be divided into two parts, including innate and adaptive immunity, based on mechanical immune responses. The innate immunity can respond to common molecules, which is pathogen associated molecular patterns (PAMPs), these molecules are conserved wide groups of microorganisms.(43) When PAMP molecules are recognized by pattern recognition receptors of immune cells, innate immunity cannot response to each type of microorganisms with specific mechanism. On the other hand, the adaptive immunity responds against the foreign substances with specific function of lymphocyte.(44) Currently, the immunology research attempts to study the mechanism of probiotics and their function associated with immunomodulation of host. There are several research proved that probiotics are able to modulate host immunity, *L. plantarum* WCFS1 could significantly promote dendritic cell penetration in the peyer's patches and enhance regulatory T cell generation.(45) Rajput IR, *et al*. demonstrated that effect of *S. boulardii* and *B. subtilis* B10 could significantly induce function of mucosal immunity via expression of TLRs and several cytokines in jejunum and ileum of broilers.(46) Llewellyn A, *et al*. showed the meta-analysis result that many probiotics can modulate innate immune responses via stimulation of micro-RNAs.(47) Additionally, peptidoglycan (PG05) of *L. rhamnosus* CRL1505 expressed the immunomodulatory effects in immunocompromised-malnourished mice after pneumonia infection by activating B lymphocyte, phagocytic activity by opsonin-like molecules and T helper 2 lymphocyte induction to exterminate *S. pneumoniae*.(48) In conclusion, probiotics have been researched to confer about immunomodulatory effect in animal and human. Some probiotics can activate the immune response, but some probiotics suppress immune response both humoral and cell-mediated immune response via different pathways to provide a beneficial effect on the host. However, there is rarely research found that probiotic could over-activate and suppress an essential mechanical pathway to resist pathogens, which is a rare case.

2.3.3 Anti-carcinogenic activity of probiotics

Cancer is a chronic disease, which occur from the growth of abnormal cell. The aggressive cancer cell can invade and metastasize to the other sites in the body from origin site. While benign tumor is a kind of cancer cells, which are besieged and localized at the origin site. Cancer has been a dreadful disease in the world in 2012, WHO had revealed the statistics of cancer that new cases are about 14 million and cancer-related deaths are about 8.2 million.(49) The mechanism of carcinogenesis is genetic mutations from environmental factors, inherited factors, and behavioral factors, these factors contribute to the oncogene activation and immune suppression. The alcohol and toxic from smoking chemicals are a common carcinogen that lead to genetic mutations. Physical agents, radiation, and infection are also the causes of tumorigenesis. Presently, molecular researches attempted to develop chemical drugs and natural substances with the potential to anti-tumor activity, but there are also several limitations, as it is long-time to develop the natural substances act as new drugs and some time they still express side effects to normal cells. There are many alternative treatments for cancer disease and one of them is probiotics administration which act as novel prevention and treatment strategy, especially tumor in the gastrointestinal tract ,as probiotics usually localize in the intestine and appear their activity in the gastrointestinal tract.(50) Several intense research involved pathology of cancer explicated about anti-carcinogenesis activity of probiotics for instance. *In vitro* studies indicated that lactobacilli exhibited high efficient to inhibit colorectal cancer cell proliferation and promote normal epithelial colon cell growth.(51)

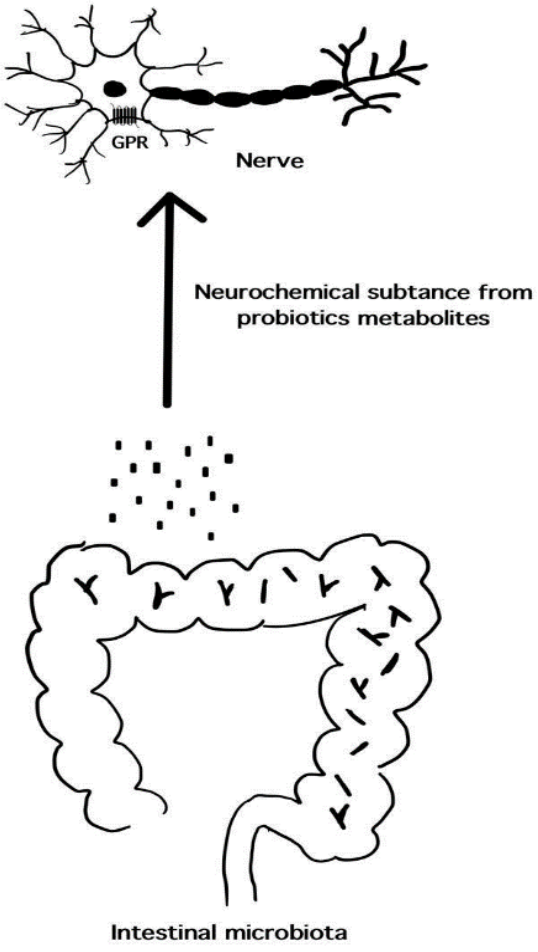
2.3.4 Anti-allergic activity of probiotics

Allergy is a kind of immune disorder which is hypersensitivity caused from over response to allergen in the environment. Allergens are harmless substance, including food, pollutant, pollen, and metals, which can stimulate immune response with overproduction of immunoglobulin E (IgE antibody). IgE antibody binds on the surface of basophils or mast cells as a receptor of allergen, when allergen pass into the body, it is at caught by IgE antibody which contributes to anaphylactic reaction from over secretion of the inflammatory cytokines. Treatment strategy for severe reaction that physician recommend is anti-histamine and steroid injections.(52) However, the basic prevention of allergy is avoidance for allergen exposure. Previously, there were many researches supported that the ability of probiotics could reduce allergic reaction. The probiotic activity of *L. rhamnosus* GG (LGG) could significantly decreased eczema when compared with placebo treatment.(53) Moreover the oral administration of *L. reuteri* could activate cytokines expression of T helper 1 and 2 cells, including IFN-γ, IL-10, TGF-β, and Foxp3 to enhance tolerogenic immune responses against food allergy in BALB/c mice model.(54)

2.3.5 Beneficial health effect of probiotics on host nervous system

The body functions are controlled by the nervous system. The autonomic nervous system mainly influences to the gastrointestinal tract with both parasympathetic and sympathetic pathways to regulate the gastrointestinal function, which is called the enteric nervous system. There are many previous evidences concluded the interaction between probiotics in the gastrointestinal tract and enteric nervous system that the gut microbiota secreted some active molecules linked with enteric nervous system to improve gut function. Active molecules are absorbed into enterocyte to stimulate signaling pathway in cytosol and nucleus which lead to expression of enterochromaffin and enteroendocrine. Nerve cells locating in gastrointestinal tract can recognize enterochromaffin and enteroendocrine through Toll-like receptor or G-protein-coupled receptor in order to enteric neural development and gastrointestinal function as shown in

Figure 2. Bercik *et al*. demonstrated that neurochemical substance releasing from *B. longum* in fermented medium could regulate myenteric nerve activity by vagal pathway to enhance digestive process.(55) Additionally, *L. salivarius* showed the secretomotor function effect to regulate the physiological gut response.(56) However, effect of probiotics on host nervous system should be further studied in order to understand more.(57)



GPR = G-protein link receptor

Figure 2 Impact of probiotics on the gastrointestinal-nervous system

2.3.6 Beneficial health effect of probiotics on metabolic syndromes

In recent years, about 25 % of the adult population in the world wide is the group of metabolic syndrome patient. Further, WHO predict about 34 % of the adult population is the group of risk factors.(58) The common causes of metabolic syndrome are genetics with environmental factor. Metabolic syndromes are associated with a health condition and metabolic factors in the patient's body, especially sugar and lipid compound metabolic pathway. Common signs and symptoms are criteria for medical diagnosis, including abdominal obesity, high blood pressure, high blood sugar, high serum triglycerides, and low high-density lipoprotein (HDL-C) levels. Insulin resistance or impaired glucose tolerance is a metabolic factor of diabetes mellitus, which can lead to hyperglycemia shock and cardiovascular complications. Lipodystrophy is a kind of pathology of metabolic syndrome associated with lipid metabolism contributing to hyperlipidemia in patient's blood circulation. There are different types of lipid disorders, including familial combined hyperlipidemia (high total cholesterol, LDL-C, triglyceride, and low HDL-C levels), familial defective apolipoprotein B-100 (high total cholesterol levels only), familial dysbetalipoproteinemia (high total cholesterol with triglyceride levels), familial hypertriglyceridemia (high triglyceride levels only), and familial hypercholesterolemia (high total cholesterol levels only). These syndromes contribute to other related diseases and health complication, especially cardiovascular and nervous system. There are many alternative treatments, which probiotics is a new strategy for therapy. Effect of probiotics on metabolic syndrome was investigated both *in vitro* and *in vivo* study, *B. lactis* HN019 exhibited an effect to reduce lipid profile, glucose, and proinflammatory cytokines, which may reduce cardiovascular risk.(59) Additionally, several LAB that showed cholesterol-lowering activity in high fed diet rat model.(60) Several probiotics could produce BSH enzyme, which contributed to decrease lipid absorption into intestinal epithelial cells, because BSH is able to convert conjugated bile salt to deconjugated bile salt, resulting in deficient absorption of lipid. Additionally, glucose and lipid assimilation activity can consume glucose and lipid in the lumen of intestine to produce their molecules and cell structure.(8) Furthermore, there is another thing coupled with probiotics, that is prebiotic because probiotics will not be able to work fully without prebiotics. The biological mechanism of probiotics to regulate lipid absorption is summarized in Figure 3.(61)

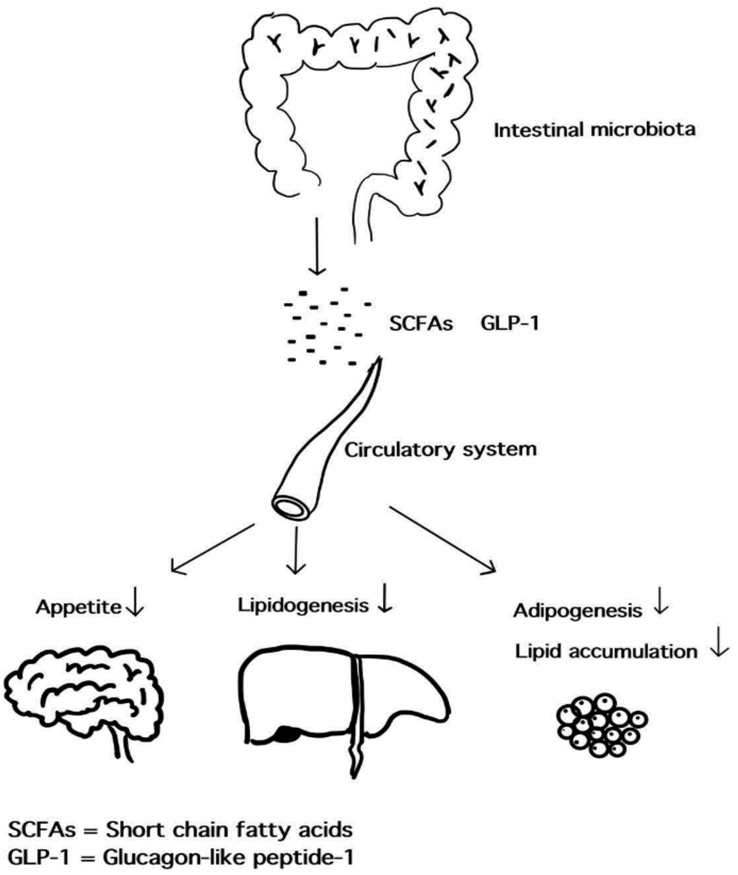


Figure 3 Probiotic mechanism exerts their lipid regulation

3 Definition of prebiotics

Prebiotics are a dietary fiber which are not digested by hydrolytic enzymes in the gastrointestinal tract, but it can be digested with probiotics in the intestine, the product of prebiotic digestion is postbiotics. Therefore, prebiotics are a food of probiotics and is useful in stimulation the growth of probiotics.(62) The nutrients that are classified as prebiotics must possess three features. Firstly, the nutrients must not be digested with the hydrolytic enzymes from the gastrointestinal tract. Second, the nutrients must be digested by probiotics. Lastly, nutrients must be beneficial effect to the host. Most of the prebiotics are usually large molecules of carbohydrates, which is commonly found in fruits and vegetables such as inulin, oligofructose, oligomannose, and so on.(3)

4 Definition of synbiotics

Synbiotics are supplement product that contain both probiotics and prebiotics. Therefore, prebiotics play an important role in enhancement the growth of probiotics. Product after the digestion of prebiotics is postbiotics that exhibit many health benefits.(63) Thus, the administration of synbiotics in sufficient amount can promote the health status rather than consume only probiotic or prebiotic products as shown in Figure 4.(64) However, synbiotic supplement has to take into account the selection of the probiotic strains to be appropriate for the type of prebiotics to maximum efficiency in promoting health benefit.(65)

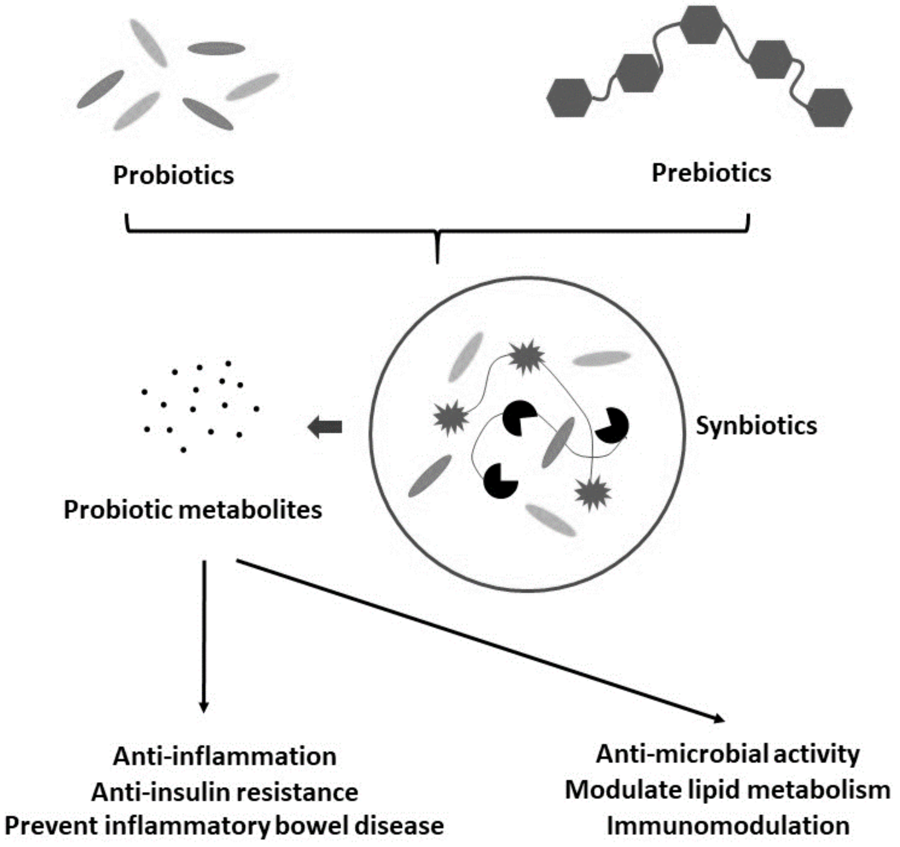


Figure 4 Schematic of probiotics and prebiotics linkage the gut microbiota

As mentioned above, probiotics and prebiotics display many beneficial effects to the body. These health issues are being studied extensively for use in daily life and the medical field. Currently, probiotics are developed into various healthy products, especially dietary supplements, because it is high potential to promote health, then it shows side effects less than chemical drugs. Medical aspect has been planned an alternative strategy to be the goal of prevention of disease rather than treatment of the disease by suppression the risk factors and enhancement the hot's immunity. Moreover, people realize on health care, as well as Thailand enters to the aging society. In 2017, number of Thai people's deaths from metabolic syndrome are 66,815 per 100,000 people.(66) Furthermore, other diseases: cerebrovascular disease, diabetes, high blood pressure, and stroke are very high incidence, which can be seen that all of these diseases are metabolic syndrome which caused from lifestyle, eating habits, and exercise. Dyslipidemia is the main cause of metabolic syndrome, especially cholesterol. Current treatment guideline for patients treatment with hypercholesterolemia is the use of drugs combined with exercise and diet control.(67) However, these treatment is still unsatisfactory, because most people have less time for exercise. Presently, probiotics have been conducted involving with medical research to develop as alternative treatment, especially non-communicable diseases, which is affecting the Thai population.(68) Probiotic supplement is medical food with convenient for use and high efficiency to promote health status. Therefore, probiotic supplement with cholesterol-lowering effect is suitable to use for prevention of metabolic disorders caused from hypercholesterolemia. Although, safety and health claim of probiotics should be considered as it may show side effect and bacteria-host interaction, even though it is the rare case.

5 Cholesterol

Cholesterol ((3β)-cholest-5-en-3-ol) comes from the Greek word that is "chole" means bile and "stereos" means solid, because the researchers found cholesterol with a solid form in gallstone. In 1815, Eugène Chevreul named this substance as cholesterine. Finally, it was called cholesterol. Cholesterol is biomolecule substance that are both lipid and alcohol properties, it can be found in the cell membrane component of all tissues in the body to balance fluidity following fluid mosaic model. It is transported in the bloodstream via lipoprotein.(69) The body can take cholesterol from food and spontaneous biosynthesis. The most cholesterol does not come with food, but it is synthesized within the body. Because the ingested cholesterol is esterified in choresteryl ester form, which is difficultly digested. For these reasons, choresteryl ester is converted to free cholesterol by cholesterol esterase in the small intestine. After absorption, free cholesterol is re-esterified to choresteryl ester by Acyl-CoA cholesterol acyl transferase (ACAT) and then it is assembly with apolipoprotein B-48 (chylomicron; ApoB-48) as the chylomicron formation to transfer to the liver, muscle as well as adipose tissues. Cholesterol in the liver is transferred to extrahepatic tissues, including blood vessel and adipose tissue by combination with apolipoprotein B-100 to produce very low-density lipoprotein (VLDL: ApoB-100) and converse to high-density lipoprotein (HDL: ApoA) by lipoprotein lipase, following by conversion to Intermediate-density lipoproteins (IDL: ApoB-100, E) and low-density lipoprotein (LDL: ApoB-100) respectively. On the other hand, free cholesterol from extrahepatic tissues is esterified to cholesteryl ester by lecithin-cholesterol acyltransferase (LCAT) and transferred to the liver by combination with apolipoprotein A to form HDL-C.(70) Therefore, LCAT enzyme plays an important role in removing cholesterol from extrahepatic tissues.(71) Recycle of cholesterol occurs from the liver secrete them into bile form to the small intestine, which is reabsorbed back to blood circulation about 50% via hepatic portal system as shown in Figure 5.(72) Cholesterol metabolism involves with the various part of the body. Additionally, function of cholesterol also relates with the physiological process of the body. Their main functions are precursor of bile synthesis, vitamin D synthesis, blood lipoprotein structure, and cell membrane structure. The excess cholesterol is excreted with feces, as bile salt is metabolized with bacteria in the gastrointestinal tract. Cholesterol in bile form is converted to non-absorbable sterol which is eliminated with feces.

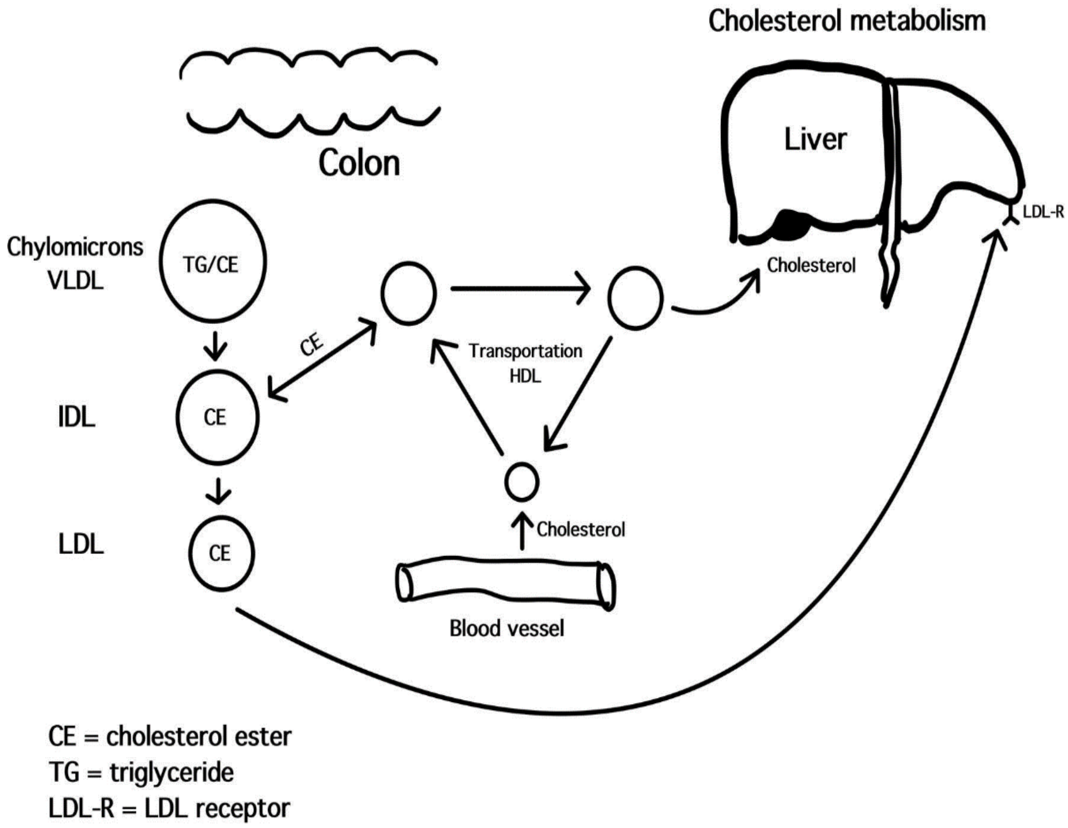


Figure 5 Cholesterol transport pathway

5.1 Cholesterol biosynthesis and their functions

Cholesterol is a type of lipid that is important with the body function. It can be synthesized through the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) pathway to convert the excess acetyl CoA to cholesterol with HMG-CoA reductase as rate-limiting enzyme. The production of total cholesterol in the body is about 20-25% (about 1 g/day) which occurs primarily in the liver. Other parts of the body that are inferior, including the intestine, adrenal gland, and reproductive organ. cholesterol plays an essential role in a cell membrane component and also as a precursor of vitamin D, bile acid, and steroid hormones.(73)

5.1.1 Cholesterol acts as a component of lipoprotein

Lipoprotein is an amphipathic complex molecule that is responsible for allowing the body to transport lipid in blood and lymphatic circulation. There are the layers of phospholipid and cholesterol locate on the outer edge, which are the hydrophilic or the water-soluble side, while the inner part of cell membrane is a hydrophobic or fat-soluble side to transport the lipid compound. There are many types of lipoprotein include: HDL-C, it is produced from the liver which plays an important role in transportation of excess fat and cholesterol back to the liver for metabolism. LDL-C, it is also produced from the liver to transport metabolic fat and cholesterol from liver to accumulate in blood vessels and adipose tissue. VLDL, it can be produced from the liver, function is responsible to transport triglyceride that the body produces from the liver to the blood vessel, adipose tissue, and skeletal muscle. And chylomicron, it is only one lipoprotein that is produced from enterocyte, it facilitates to transport triglyceride from the small intestine to liver and adipose tissue.(74) All of lipoproteins exhibit their function in relation to control lipid level to suit for the body requirement.

5.1.2 Cholesterol acts as a precursor of vitamin D synthesis

Vitamin D (calciferol) is a type of fat-soluble vitamin that performs important function to regulate calcium absorption as well as bone calcification. Vitamin D can be produced from cholesterol in the dermal layers, caused from 7-dehydrocholesterol reacts with sunlight, it is converted to vitamin D_3 (cholecalciferol). After that, vitamin D_3 is transported to the liver and converted to 25-hydroxycholecalciferol. In the kidney, 25-hydroxycholecalciferol is converted to 1,25-dihydroxycholecalciferol which it is active form to stimulate calcium absorption and modulate calcium concentration in blood circulation.(75)

5.1.3 Cholesterol acts as a precursor of sex hormones synthesis

The body can produce sex hormones, including testosterone, estrogen, and progesterone from the adrenal cortex and sexual organs. The kidney is considered an important organ for the production of sex hormones. Cholesterol in blood circulation acts as a precursor of sex hormone by converting testosterone hormone which later changed to a progesterone and finally became an estrogen.(76)

5.1.4 Cholesterol acts as a precursor of bile acid synthesis

Bile is a bitter greenish yellow liquid secreted from the hepatocyte in the liver of almost all vertebrates. Bile of several animals is stored in the gallbladder, when eating gallbladder is stimulated to release bile into the duodenum and then bile acts as an emulsifier to facilitate lipid digestion. The end product of cholesterol utilization is bile acid which liver is organ that completely synthesizes bile acid.(77) Initially, the hydroxylation of cholesterol is performed by cholesterol 7α-hydroxylase (CYP7A1) in the classical pathway or sterol 27- hydroxylase (CYP27A1) in the alternative pathway, which are rate limiting step in bile acid synthesis. Next, 7α-hydroxycholesterol is converted to chenodeoxycholic (45%) acid and cholic acid (31%) by sterol 12α-hydroxylase (CYB8B1). These are referred as the primary bile acids, which they are added with coenzyme A (CoASH) to convert as cholyl CoA. Cholyl CoA is an unconjugated bile acid, which is poorly water-soluble because it present pK of 6 and their structure is mostly hydrophobic. After that, cholyl CoA is secreted into the canalicular lumen, it is conjugated with amide bond formation of glycine or taurine in order to convert as glycocholic acid and taurocholic acid. These are referred as the secondary bile acid and claim to bile salt, as they show pK of 3 which contribute to revealing charge in their structure. Moreover, the conjugation process increases the amphipathic effect leading to increase water-soluble property as shown in Figure 6. Finally, bile salt is secreted from the liver to gall bladder, when the chyme translocates into duodenum, cholecystokinin (CCK) hormone is secreted to promote contraction of the gall bladder. Finally, bile salt is secreted into the duodenum to facilitate absorption of lipid by emulsification processes.(78) Bile salt can bind both fat droplet and lipase to initiate lipid digestion. After that, almost 95% of bile salt is taken up from brush border epithelial cell of intestine to the liver through hepatic portal system. While about 5% is hydrolyzed with enzyme of intestinal bacteria which is excreted with feces in stercobilin form. Additionally, urobilinogen can be reabsorbed by kidney, it can be eliminated along with urine as an urobilin form.

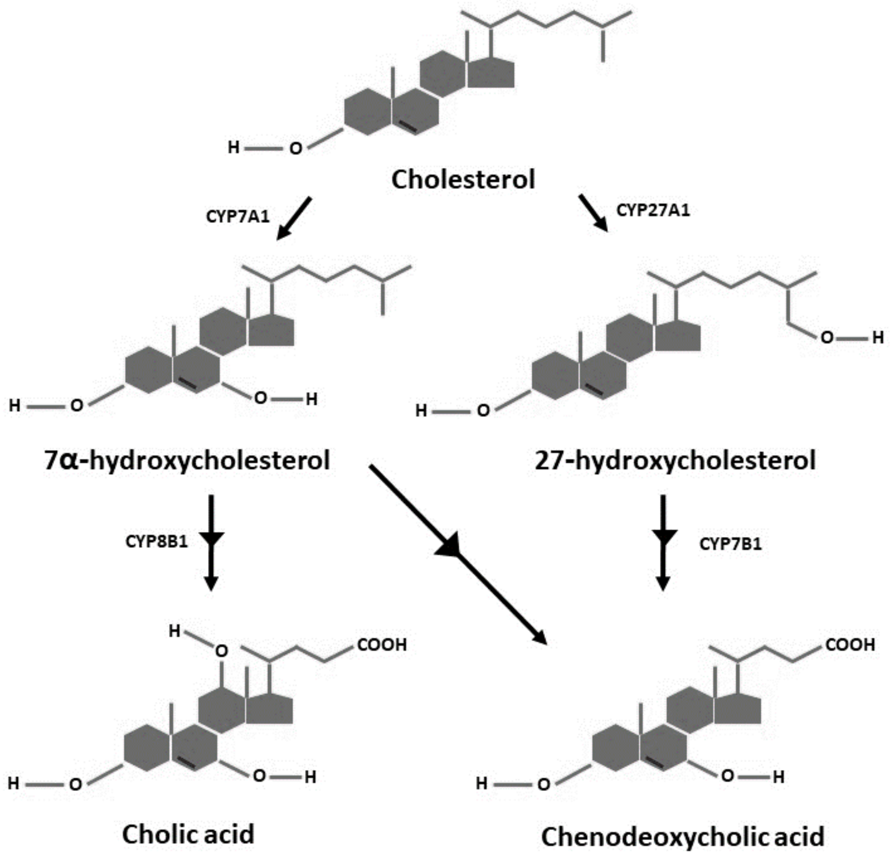


Figure 6 Bile acid synthesis

5.2 Diseases associated with cholesterol

Cholesterol is a kind of essential biomolecule to support physiological process of the body, but if the body accumulates cholesterol, it contributes to pathological effect of various diseases. There are many factors that can affect blood cholesterol levels, including lifestyle, such as smoking, consumption behavior, lacking of exercise, hormonal effects, family history, in current disease, genetics, as well as immune status.(79) There is plenty of evidence to show that high cholesterol levels or hypercholesterolemia is a common risk factor of various diseases, including stroke, cardiovascular disease, heart attack, transient ischemic attack, and peripheral arterial disease. For prevention and treatment, the modification of lifestyle and exercise are the important guidelines to plan to moderate cholesterol to normal levels. Medication is important, there are many different types of cholesterol-lowering drugs. The physician can advise on the most appropriate treatment, statins are commonly used to treat hypercholesterolemia. This drug acts to inhibit the activity of liver enzymes in cholesterol biosynthesis pathway. Ezetimibe, which inhibits the absorption of cholesterol from foods within the intestine, but this drug does not act as much as statin.(80) Presently, the new strategy of treatment is the alternative treatment, natural substances therapy can be used for treatment of hypercholesterolemia. Probiotics is considered as a kind alternative treatment because it is relatively high effective for health promoting potential without side effect, when compared to the use of antibiotics and synthetic chemotherapy drugs. Additionally, probiotics can be found as the gut microbiota in human and animal, which are individual profiles to support metabolism of host. Therefore, administration of probiotics is an alternative approach to promote health status, prevent the diseases, and promote the effectiveness of treatment.

6 Mode of action of cholesterol-lowering probiotics

One of the significant properties of probiotics is the prevention of metabolic disorders which caused by abnormal levels of lipid, especially cholesterol. There are various mechanisms of cholesterol-lowering effect by probiotics, including production of enzyme and direct assimilation.

6.1 Direct mechanism of cholesterol-lowering probiotics

The direct mechanism is cholesterol assimilation, probiotics established in the gastrointestinal tract can absorb free cholesterol in the intestinal lumen, leading to reduce cholesterol absorption of enterocyte as shown in Figure 7. Moreover, probiotics can utilize cholesterol to produce ATP by anaerobic metabolism and create their cell structure, especially cell membrane component. Previous studies revealed that several probiotic strains were tested the assimilation of cholesterol, resulting of the experiment showed that several probiotics could significantly reduce cholesterol from culture media to synthesize cell membrane.(81)

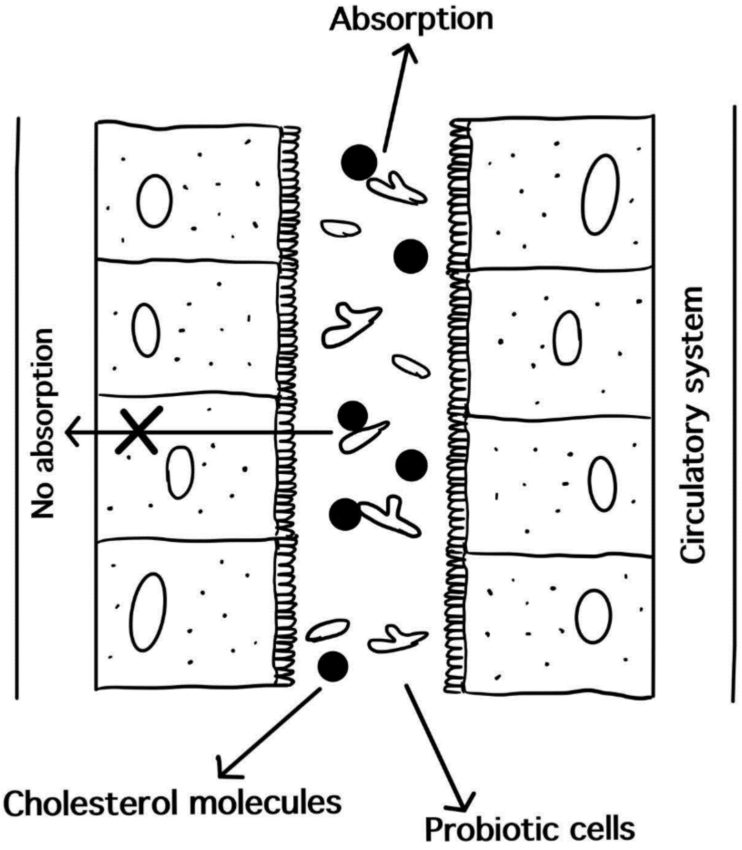


Figure 7 Mechanism of cholesterol assimilation of probiotics in the intestine

6.2 Indirect mechanism of cholesterol-lowering probiotics

Indirect mechanism is BSH enzyme activity, some probiotic strains are able to produce BSH that exhibit the effect of decomposing bile from the conjugated bile salt to deconjugated bile salt.(82) The deconjugated form is poorly soluble and excreted with fecal route, which cause of deficient the emulsification of lipid in the intestine, following to decrease the absorption of lipid into blood circulation. Additionally, probiotics can secrete some substances that have a balanced effect of cytokine, which facilitate to maintain the level of lipid and sugar in blood to prevent related diseases. Moreover, the other effects of bile salt degradation is stimulation bile acid synthesis which contributes to cholesterol reduction.(83) The previous studies showed effect of intestinal probiotics could produce BSH and reduce hypercholesterolemia. *S. cerevisiae* ARDMC1 was improved that it can reduce cholesterol in rat model.(84) While administration the other probiotics from the commercial product can also decrease cholesterol levels.

7 Bile salt hydrolase enzyme

Bile salt hydrolase enzyme (EC 3.5.1.24) is encoded from *bsh* genes, which can be founded in some probiotic species. BSH belongs to the choloylglycine hydrolase family and N-terminal nucleophile (Ntn) with an N-terminal cysteine residue superfamily of enzymes. The active site of BSH is formed from the cysteine within conserving **αββα** core of enzyme, it is called catalytic center. It facilitates to form a tetrahedral intermediate between the **αββα** core and the amide bond of conjugated bile acid. BSH mechanism can hydrolyze amide bond of alkyl group (R) of glycine and taurine-conjugated bile acid from the sterol core of cholic acid and chenodeoxycholic acid to convert as the deconjugated bile acid, as shown in Figure 8. Thus, BSH enzymes are commercially used in research aspect such as *Clostridium perfringens* BSH (CpBSH), *Bifidobacterium longum* (BlBSH), *Lactobacillus salivarius* (LsBSH), *Enterococcus faecalis* (EfBSH), etc.(85)

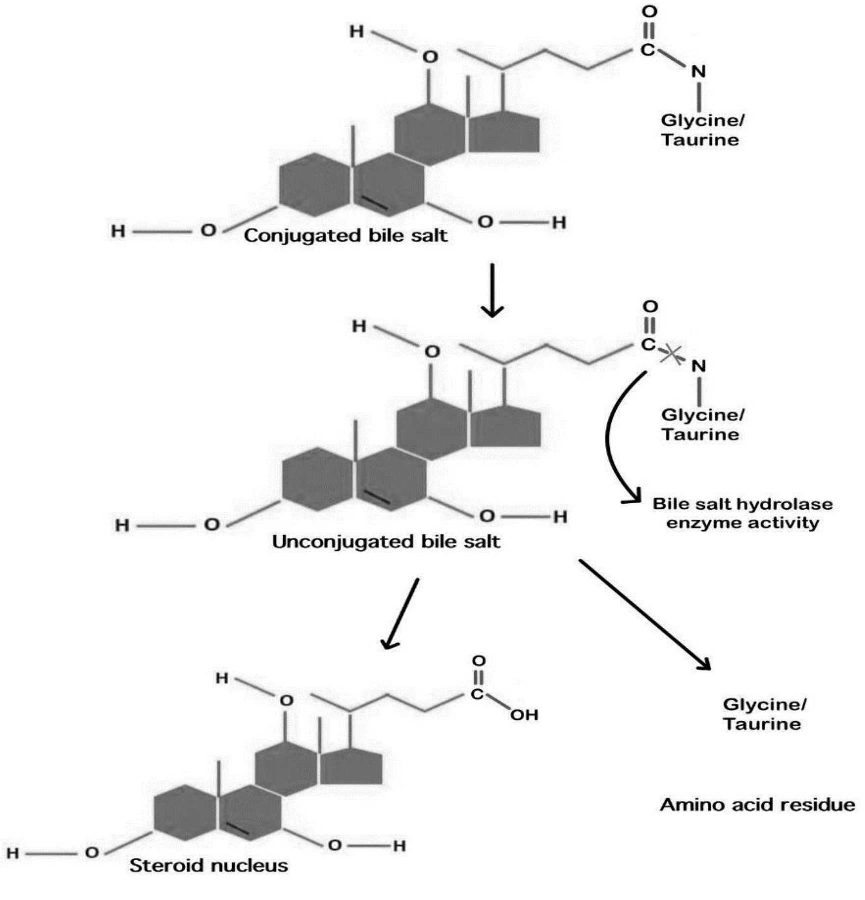


Figure 8 Mechanism of bile salt hydrolase enzyme

Currently, probiotics have been developed into various probiotic products because they can express various health promoting effects. The development of probiotic products that consist of many probiotics can synergize several properties of probiotics in the products. Moreover, probiotic products can develop in a synbiotic product to promote the growth of probiotics in the body after consumption. However, industrial processes in the production of probiotic products have many factors that affect to viability and stability of probiotics during production, storage, transportation, and distribution of products. These factors include temperature, moisture, acidity (pH), osmotic pressure and the effect of various mechanical forces. Additionally, the gastrointestinal environment also affects the survivability and growth of probiotics in the body after passage through the gastrointestinal tract. These factors include gastric acid, bile condition, and various hydrolytic enzymes in the gastrointestinal tract.(86) Therefore, the microencapsulation technique have been applied to use in the production processes for enhancement the survivability and stability of probiotic products.

8 Microencapsulation

Microencapsulation is a bio-technological process to apply in several industrial processes. It is a process which uses biomaterial to encapsulate the desired substance, including vitamins, medicines, organic compounds, and probiotics, as this process can produce microcapsule size of 1 to 1,000 um. There are many advantages of microcapsules, including protection of substance property, preservation of substance or viability, delivery substances to target organ, convenient for use, improvement the shelf life of substance, as well as regulation of substance releasing to the desired area at a suitable time. Therefore, the microencapsulation is applied in various manufacturing industries. For example, an application of the microencapsulation in agriculture to control the release of pesticides that are encapsulated in microcapsules by gradually releasing, which contribute to reduction the amount of pesticides residues in the environment. An application of the microencapsulation in the medical and pharmaceutical aspect, there are many researches of microencapsulation to apply in the pharmaceutical and vaccine production, as it can delivery drug to the target organ with suitable time.(87) The structure of the microcapsules consists of 2 parts: first part is encapsulated substances that are liquid or solid texture, which are called the core or internal phase such as various vitamins, minerals, medicines, pesticides, probiotics, and so on. Second part is wall material that is usually thin wall, which is called sheath or external phase.(88) Wall material is the encapsulating matrixes or biopolymers, which is important in determining the characteristics and features of the microcapsules to be as desired. Several types of encapsulating materials that are commonly used in microencapsulation process such as gum arabic, maltodextrin, whey protein, fibersol, alginate, starch, etc. There are various forms of microcapsules include mononuclear, multinuclear and coated multinuclear, depend on the microencapsulation technique.(89) However, encapsulating materials should be the good features that can be spread as a thin film, flexible, non-reaction with core material as well as low viscosity. The process of microencapsulation has to consider many factors that play a significant role in the properties of the microcapsules. The first importance is the selection of the type of encapsulating materials, as there is different releasing mechanism of the encapsulating materials, based on pH, temperature, pressure, and enzyme as shown in Figure 9.(90)

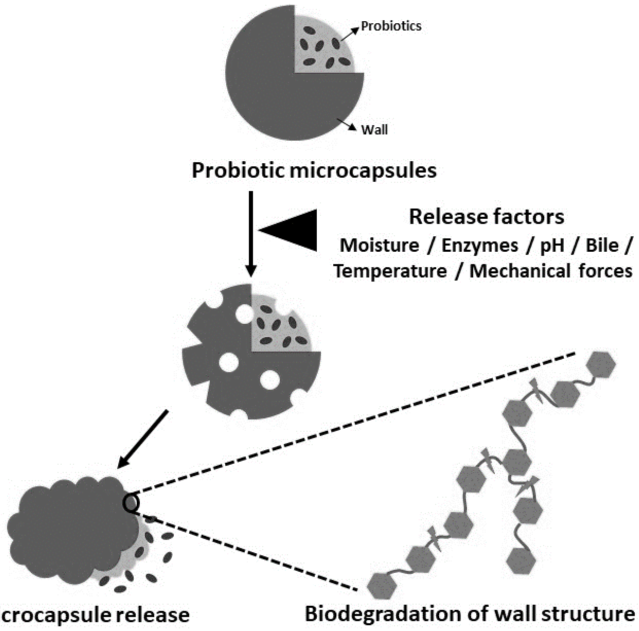


Figure 9 Releasing mechanism of the encapsulating material

8.1 Starch and nano-crystalline starch from cassava

Starch is a polysaccharide (polysaccharide) which is found in plants such as cereal grains, tuber crops, beans, etc. The structure of starch consists of glucose as a monomer connected with alpha 1,4 glycosidic bond also known as amylose, while alpha 1,6 glycosidic bond is amylopectin which generate branch within structure. These molecules are arranged to starch granules and deposit as energy source in several plants. Naturally, the starch granules of each plant contain amylose and amylopectin, which are packed with different proportions.(91) Starch in the nature form is deposited in starch granules, which its structure can be found in a semi-crystalline, including crystalline region and the amorphous region as shown in Figure 10. Crystalline region is the arrangement of amylose and amylopectin with the orderly bond, as the short chain of amylopectin is organized in a double helix structure. The amorphous region of starch granules is the arrangement of amylose and long chain of amylopectin with disorderly bond. Chemical property of crystalline region in the starch granule is able to from bond with alkyl group of protein, as their structure reveal a plenty of hydroxyl group, which can easily link bond to other functional groups of organic compounds. The content of the crystalline region in starch granules is different, based on type of starch in each plant.(92) Cassava is an important crop of the world and also economic crop of Thailand. The scientific name of cassava is *Manihot esculenta* Crantz. The common names are Cassava, Yuca, Mandioa, Manioc, and Tapioca. Cassava starch is a product derived from cassava, which is a fine white powder, high purity (95% starch and less than 1% protein and fat) as well as high content of starch granule. The morphology of the starch granule from cassava starch when examined under the microscope is a round or oval shape with dent color in the middle of granule and their size are about 3-20 um. Additionally, cassava starch contains relatively low amylose, making the structure of the crystalline region in a certain amount. Therefore, the cassava starch is widely used as an encapsulating material, as it exhibits suitable property for the microencapsulation process, including the ability to inflate and absorb water, the ability to emulsify with other encapsulating materials, the ability to gelatinize, the ability to retrograde, and especially, the ability to modify of structure.(93) As for microencapsulation process, the modification of starch is an important procedure, because it increase durable property of microcapsule during passage through the production process. The nano-crystalline starch (NCS) can be prepared from modification of cassava starch by acid hydrolysis. It has been used quite a lot in the microencapsulation process because the starch with the relatively high crystalline region can cross link the covalent bond with the other encapsulating materials, especially alkyl group of protein. Accordingly, this process contributes to the structure of microcapsules able to resist the digestion of various enzymes during the production process. The cassava starch also serve as prebiotic property, which is suitable for use in microencapsulation process of probiotic production in order to deliver probiotics passage through the gastrointestinal tract and promote the growth of probiotics. However, the cassava starch is carbohydrate which should encapsulate together with the other encapsulating materials to enhance durability and rigidity of the microcapsule, protein is suitable for use with carbohydrate.(94) Present study we select WPI as encapsulating material combines with NCS.

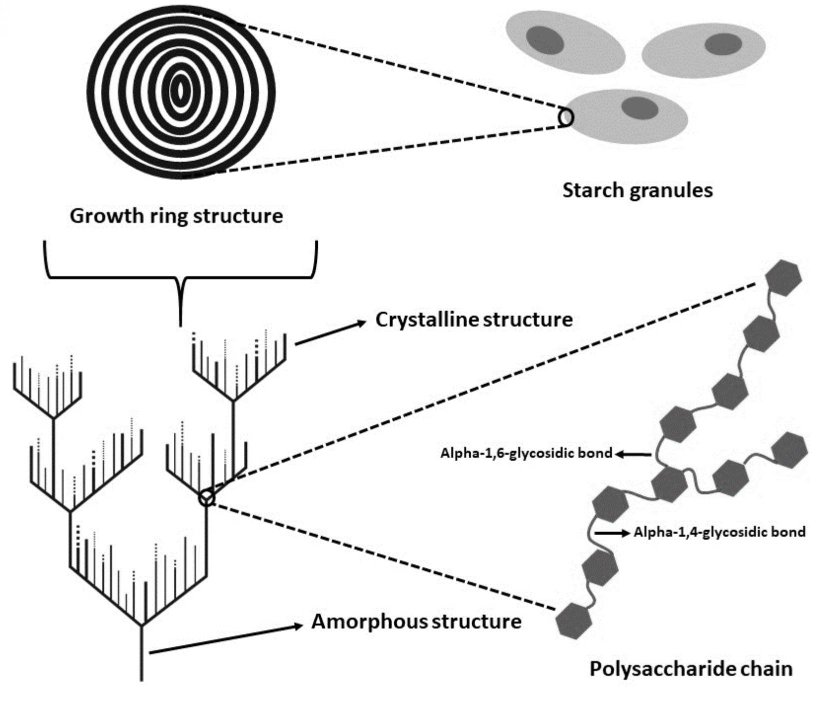


Figure 10 Structure of starch granule

8.2 Whey protein and various products of whey protein

Whey protein is a protein isolated from milk product, which is a high quality protein and the body can digest and absorb easily. Whey protein contains essential amino acids, especially branched-chain amino acid. Thus, whey protein constitutes high nutrition and promote health, as it is important to strengthen the muscle and other tissues during growth. The crucial component of whey protein is beta-lactoglobulin, alpha-lactalbumin, and albumin, other than there are also minor proteins and minerals such as glycomacropeptide, lactoferrin, lactoperoxidase, relaxin, calcium, iron, etc. Manufacturing process of whey protein is precipitation of protein in the cheese production after separating casein and fat, which is clear light yellow liquid. Whey protein can be processed into various products, based on separation techniques.(95)

8.2.1 Whey protein concentrate

Whey protein concentrate is produced from whey protein solution by passage through the membrane ultrafiltration process to separate lactose and milk fat. After that, it is dehydrated with a spray dryer for forming of whey protein concentrate powder. This product contains whey protein concentration less than 90% and the texture is a light creamy color with natural milk flavor.(96)

8.2.2 Whey protein isolate

WPI is a pure whey that is produced from the whey protein concentrate by increasing the concentration of whey protein more than 90%, while the concentration of lactose and fat until remain. Ion-exchange process is used to separate other molecules with different electric charges in each molecule. The Ion-exchange process can produce pure whey protein as much as possible, which is relatively high whey protein concentration of 98%. The texture of WPI appears light creamy powder with natural milk flavor.(97)

8.2.3 Hydrolyzed whey protein

Hydrolyzed whey protein is WPI or whey protein concentrate that are hydrolyzed the molecule of whey protein to be oligopeptide and free amino acid. The advantages of hydrolyzed whey protein are enhancement for absorption and reduction of protein intolerance.(98)

As described above, WPI exhibits the highest protein concentration as well as purity. As for microencapsulation process, WPI is mostly suitable for use as encapsulating materials with NCS because the high protein content in the molecule increases the ability to cross link by intermolecular glycosylation with reducing sugar molecules of NCS. Thus, microcapsule wall is durable and flexible, which it is suitable to use for spray drying microencapsulation of the desired substances to improve the survivability and stability of probiotics.

8.3 Types of microencapsulation techniques

There are several techniques of microencapsulation were applied to use in industrial process of probiotic production. Each technique is able to produce microcapsule with different sizes and structural textures. Therefore, the selection of microencapsulation techniques is an important criteria in determining the characteristics of microcapsules.(99)

8.3.1 Chemical microencapsulation techniques

8.3.1.1 Phase separation or coacervation technique

Phase separation or coacervation is widely used for preparation of microcapsule with long term method. The principle of this technique is changed the solubility of colloidal system, as phase of core substance is mixed with phase of shell substance to form wall structure with durability and stability. However, this microencapsulation technique have to control the ratio of mixture to be stable with consistent for production of microcapsule with round shape.(100)

8.3.1.2 Polymerization technique

The principle of this technique is the polymerization of monomer or shot polymer on the surface of the microcapsule to encapsulate the core substance. The preparation initiates the dissolution of the monomer in the water phase with the dispersant core substance. Then add the organic compounds to accelerate the polymerization of monomer in order to create a microcapsule wall. Therefore, core substance is imbedded in the structural polymer of microcapsule wall. The advantages of this technique are able to produce small microcapsule with low particle size distribution and does not require heat, resulting in the stability of the core substance.(101)

8.3.1.3 Emulsion technique

Emulsion technique has been commonly applied for microencapsulation of probiotics, this technique requires the continuous phase for emulsion. Briefly, a small volume of a desired substance with coating material (dispersed phase) is mixed into the large volume of vegetable oil (continuous phase) by using an emulsifier in order to form the water-in-oil phase. Once water-in-oil phase formed, microcapsule (soft gel) will be produced in the continuous phase after adding calcium chloride solution, as calcium chloride can stimulate lattice formation of microcapsule wall during stir the solution. The size and shape of microcapsule depend on the stirring speed of solution and the proportion of the two phases. Alginate is a encapsulating material that is recommended for this technique to produce microgels.(102)

8.3.2 Physical microencapsulation techniques

8.3.2.1 Extrusion technique

Encapsulation technique is commonly applied to encapsulate the volatile organic compound. The encapsulating mixture is controlled to extrude through the nozzle to determine the size of microcapsule. Furthermore, this technique requires calcium chloride solution. After dripping, the encapsulating material is immediately harden, as it synthesizes lattice formation to overlay the core inside. Thus, the extrusion technique is a true microencapsulation process, because volatile substances are completely coated with an encapsulating material.(103)

8.3.2.2 Spray drying techniques

Spray drying techniques are the method to use spraying for the production of dry microcapsule. The core substance is essential to disperse in the encapsulating solution. This mixture is sprayed into the drying chamber or cooling chamber in order to evaporate the solvent from heat in the spray drying process or solidify the microcapsule wall from the freezing cold (spray congealing), this step contributes to the core substance is encapsulated in the encapsulating material. After that, the microcapsule will be air blown and accumulated in the collecting chamber. This technique is suitable to use for production of drug and probiotics, as it can be used with unstable substances and microorganisms because it produces microcapsule in dry form that can inhibit the chemical reaction and metabolism within microorganisms. The spray drying technique can produce microcapsule in fine powder form, but the diameter of particle size distributes within the range of 10-100 um.(10) Additionally, the heat labile substances, including vitamins and organic compound can be used with spray congealing technique. However, the encapsulating material that is suitable for spray drying technique should be the thermally-resistant materials.(104) However, the use of this technique should be considered factors which affect to the characteristics of microcapsule, including viscosity of the sprayed substance, the temperature in the drying section, speed of spray, and air pressure.(105)

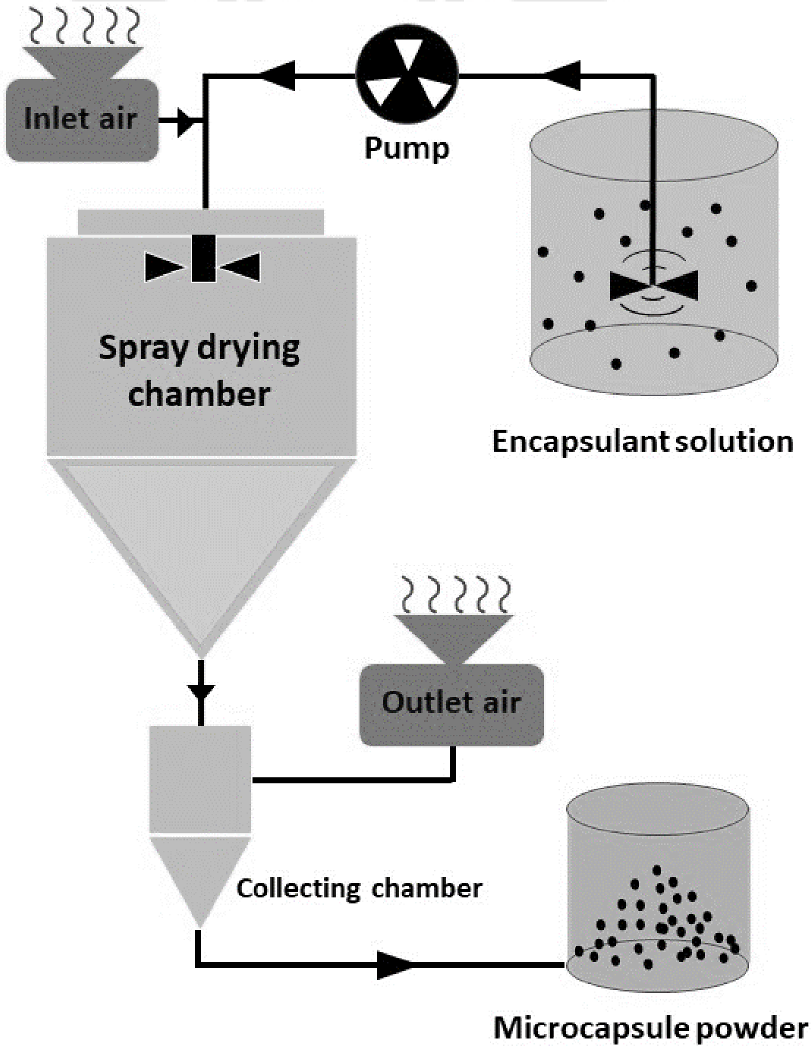


Figure 11 Schematic diagram of spray drying microencapsulation

Table 3 Comparision of microencapsulation techniques

Microencapsulation	Materials	Scalability	Product	Application
Coacervation	Polysaccharide, Protein	100-500 um	Microgels	Gel tablet (Drug delivery)
Polymerization	Polysaccharide	10-100 um	Microgels	Gel tablet
Emulsion	Polysaccharide, Lipid	100-1,000 um	Microgels	Gel tablet (Drug delivery)
Extrusion	Polysaccharide, Protein	100-500 um	Microgels	Gel tablet
Spray drying	Polysaccharide, Protein	10-100 um	Dry powder	Brew powder

As described above, probiotic LAB are able to express various health promoting effects. Additionally, probiotic LAB constitute in the gut microbiota with different in each individuals and high diversity. Thus, they can synergize their activity to promote health status, especially modulation the metabolism of nutrient in the host' gastrointestinal tract. BSH enzyme is a kind of probiotic activity that regulate the metabolism of lipid which contributes to modulate cholesterol metabolism. Thus, cholesterol-lowering probiotic LAB are interested to develop as cholesterol-lowering probiotic products. However, the production of probiotics for use as supplement product often encounters problems with viability and stability of probiotics. Microencapsulation is a biotechnological process, which is used to develop for protection of probiotic cells from the various stress conditions. Therefore, the aims of this study were to develop cholesterol-lowering probiotic products from three genera of probiotics LAB with ability to reduce cholesterol by BSH activity and cholesterol assimilation, and functional characteristics of probiotics, including *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. via spray drying microencapsulation with WPI and NCS in order to enhance the survivability and stability of probiotic LAB during passage through the gastrointestinal tract and long term storage, respectively. Consequently, cholesterol-lowering probiotic LAB could reduce cholesterol levels and establish as gut microbiota after administration, which contribute to prevention the risk of metabolic disorders caused by hypercholesterolemia and providing synergistic effect of their health promoting potentials.

CHAPTER 3

MATERIALS AND METHODS

Chemicals and materials

1. De Man Rogosa Sharp (MRS) media (HiMedia, India)
2. Bifidobacterium selective media (BSM) (HiMedia, India)
3. Blood agar base (HiMedia, India)
4. Mueller Hinton agar (MHA) (HiMedia, India)
5. Bifidobacterium selective supplement (HiMedia, India)
6. Eosin Methylene Blue agar (EMB) (HiMedia, India)
7. Simmon's citrate agar (HiMedia, India)
8. Skim milk (HiMedia, India)
9. Starch agar (HiMedia, India)
10. Egg yolk agar (HiMedia, India)
11. Motility test medium (HiMedia, India)
12. Anaerobic gas package (MGC, Japan)
13. Sodium salt of taurodeoxycholic acid (TDCA) (Sigma-Aldrich, USA)
14. Calcium chroride ($CaCl_2$) (Merck, Germany)
15. Calcium carbonate ($CaCO_3$) (Merck, Germany)
16. Ethanol (C_2H_6O) (Merck, Germany)
17. Sodium hydroxide (NaOH) (Merck, Germany)
18. Hydrochloric acid (HCl) (Merck, Germany)
19. Acetic acid ($C_2H_4O_2$) (Merck, Germany)
20. Sulfuric acid (H_2SO_4) (Merck, Germany)
21. Paraformaldehyde ($HO(CH_2O)_nH$) (Merk, Germany)
22. Isofurane ($C_3H_2ClF_5O$) (Piramal clinical care, USA)
23. Glycrol ($C_3H_8O_3$) (Merck, Germany)
24. Cholesterol (Sigma-Aldrich, USA)
25. 1-Butanol ($C_4H_{10}O$) (Merck, Germany)

26. Hydrogen peroxide (H_2O_2) (Merck, Germany)
27. Tris-HCL (Merck, Germany)
28. Oxgall or bovine bile (Sigma-Aldrich, USA)
29. Trypsin (Sigma-Aldrich, USA)
30. Pepsin (Sigma-Aldrich, USA)
31. Gamma-amino butyric acid (GABA) (Sigma-Aldrich, USA)
32. Glutamic acid (Sigma-Aldrich, USA)
33. Monosodium glutamate (Sigma-Aldrich, USA)
34. TLC plates, Silica gel 60 F_{254} (Merck, Germany)
35. Ninhydrin spray solution (Merck, Germany)
36. Antibiotic drugs (Becton Dickinson, USA)
37. O82G/15 C.P. MICE FEED (National Laboratory Animal Center of Mahidol University, Bangkok, Thailand)
38. Cell lines: Human colon adenocarcinoma cell line (Caco-2) (ATCC, HTB-37)
39. Dulbecco's Modified Eagle’s Medium (DMEM) (Gibco-invitrogen, USA)
40. Fetal bovine serum (FBS) (Gibco-invitrogen, USA)
41. Penicillin-Streptomycin (Gibco-invitrogen, USA)
42. 25% Trypsin (Gibco-invitrogen, USA)
43. Dimethyl sulfoxide (C_2H_6SO) (DMSO) (Fisher Scientific, India)
44. Triton® X-100 (Merck, Germany)
45. Trypan-blue (Gibco-invitrogen, USA)
46. Tapioca starch (Siam Quality Starch, Thailand)
47. Whey protein isolate (Vicchi Enterprise, Thailand)
48. Agarose powder (HiMedia, India)
49. 50x Tris-borate-EDTA (HiMedia, India)
50. 1kb DNA ladder (HiMedia, India)
51. OnePCR™ Ultra (completed master mix) (GeneDirex, USA)
52. HiPurA™ Bacterial Genomic DNA Purification Kit (HiMedia, India)
53. PCR Clean-Up & Gel Extraction Kit (GeneDirex, USA)

54. Hemacytometer (Thermo Scientific, USA)
55. ELISA plate: 96 well plate (Nunc Maxisorp, USA)
56. 6-well tissue culture plate (Corning, USA)
57. 24-well tissue culture plate (Corning, USA)
58. Disposable sterile serological pipette (Corning, USA)
59. 0.22 um membrane filter (Merck, Germany)
60. 0.45 um membrane filter (Merck, Germany)
61. Tissue culture flask (Corning, USA)
62. Conical centrifuge tube (Corning, USA)
63. Cryovial (Nalgene, USA)
64. Syringe (NIPRO, Thailand)
65. C1000™ Thermal Cycler (Bio-Rad, USA)
66. NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA)
67. Spectrophotometer (Shimadzu, Japan)
68. BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA)
69. PowerPac™ Basic Power supply (Bio-Rad, USA)
70. Gel electrophoresis chamber MiniRun GE-100 (Handzhou Bioer technology, China)
71. Autoclave (J.P. Selecta, Spain)
72. Water bath (J.P. Selecta, Spain)
73. Whitley Jar Gassing System (Anerocult, UK)
74. Biosafety cabinet (Nuaire, USA)
75. Anaerobic jar (Mitsubishi, Japan)
76. Light microscope (Olympus, Japan)
77. pH meter (Thermo Scientific, USA)
78. X-ray diffractometer (Bruker, Germany)
79. Scanning electron microscope (Hitachi High-Technologies, Japan)
80. Freeze dryer (SciQuip, UK)
81. Incubator shaker (Labnet International, USA)

82. Incubator (J.P. Selecta, Spain)
83. High speed centrifuge (KUBOTA, Japan)
84. Centrifuge (Sartorious Stedim, Germany)
85. Magnetic stirrer (IKA, Germany)
86. Mini Spray Dryer B-290 (Buchi, Switzerland)
87. Desiccator (Thomas Scientific, USA)

Methods

1 Functional characterization of probiotic lactic acid bacteria with bile salt hydrolase activity

1.1 Isolation and selection of probiotic lactic acid bacteria

Traditional foods were taken from several markets in Chachoengsao province. During transportation, all samples were stored at 4˚C for preservation. De Man Rogosa Sharpe media (MRS; HiMedia, India) and *Bifidobacterium* selective media (BSM; HiMedia, India) were prepared to isolate probiotic lactic acid bacteria (LAB). All samples were inoculated into 5 mL of MRS broth under anaerobic incubation at 37 ˚C for 48 h, then bacterial culture was sub-cultured onto MRS agar with calcium carbonate ($CaCO_3$) and BSM agar with *Bifidobacterium* selective supplement (HiMedia, India) until pure culture. After that, bacterial colony with ability to produce lactic acid was picked up to study gram and catalase reaction. Probiotic LAB isolates with gram positive and catalase negative were stored in MRS broth supplemented with 40% (v/v) glycerol (Merck, Germany) and stored at -80 ˚C for experimental use.

1.2 Motility assay

Motility test medium (HiMedia, India) was used to determine the ability of bacterial motile using cilia or flagella, which indicate virulence factor. Probiotic LAB isolates were grown in MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 48 h. After that, they were stabbed into Motility test medium. After anaerobic incubation, bacterial colony with ability to motile was interpreted as a positive result. *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* DMST 20654 were used as positive and negative control, respectively.

1.3 Qualitative investigation of bile salt hydrolase activity using a spot plating technique

Conjugated form of taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA; Sigma-Aldrich, USA) were used as substrate to determine bile salt hydrolase (BSH) activity. Probiotic LAB isolates were grown in MRS broth under anaerobic incubation at 37 ˚C for 24 h. After that, they were adjusted to 10^9 CFU/mL with phosphate buffer saline (PBS, pH 7.2) and spotted onto MRS agar supplemented with 0.5% (w/v) either TDCA or GDCA and 0.37 g/L of calcium chloride ($CaCl_2$) followed by anaerobic incubation at 37 ˚C for 72 h. After incubation, bacterial colony with precipitated zone of bile salt (deconjugated form) was interpreted as a positive result. *Enterococcus faecium* F23 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively.

1.4 *In vitro* cholesterol assimilation

Cholesterol assimilation was performed to measure the cholesterol utilization of probiotic LAB isolates. Probiotic LAB isolates were grown on MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 24 h. Then they were adjusted to 10^9 CFU/mL and then they were cultured into modified MRS broth containing 0.3% (w/v) bovine bile (Sigma-Aldrich, USA), 0.1 g/L water-soluble cholesterol (Cholesterol–PEG 600; Sigma-Aldrich, USA) followed by anaerobic incubation at 37 °C for 24 h, which uninoculated medium was used as control. As for cholesterol measurment, *O*-phthalaldehyde method was performed to determine cholesterol content. Two milliliters of *O*-phthalaldehyde (Sigma-Aldrich, USA) were added into sample reaction with 95% (v/v) ethanol, 33% (w/v) potassium hydroxide (KOH) and hexane, then the solution was rested at 25 °C for 10 min before adding with 1 mL of sulfuric acid (H_2SO_4). After that, the optical absorbance was measured at 550 nm. Cholesterol assimilation was calculated as percentage according to the equation:

$$\text{Cholesterol assimilation (\%)} = \left(\frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}}\right) \times 100$$

1.5 Genotypic identification of selected probiotic lactic acid bacteria using next generation sequencing

1.5.1 Amplification of 16s ribosomal RNA gene

16s ribosomal RNA (16s rRNA) gene is a target sequence of polymerase chain reaction (PCR) using the universal primers, including 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Probiotic LAB isolates were grown in MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 24 h. As for PCR, probiotic LAB cells were lysed and extracted their DNA using HiPurA^{TM} Bacterial Genomic DNA Purification Kit (HiMedia, India). The PCR master mix was prepared in a total volume 25 mL/reaction consisting of 23 uL of milliQ water, 25 uL of OnePCR^{TM} *Plus* pre-mixed solution (GeneDirex, USA), 1 uL of each primer and 1 uL of DNA template. The 16s rRNA gene amplification was performed by C1000^{TM} Thermal Cycler (Bio-Rad, USA) under the following PCR conditions consisting of 5 steps: a pre-denaturation at 94 ˚C for 2 min; 32 cycles of denaturation at 94 ˚C for 1 min, annealing at 50 ˚C for 40 second, extension at 72 ˚C for 1 min; and a final extension at 72 ˚C for 5 min. PCR product was determined by using agarose gel electrophoresis at 385 mA, 75 V for 40 min comparing with DNA ladder (Thermo Fisher Scientific, USA).

1.5.2 Identification of 16s ribosomal RNA gene

Purification of PCR product was performed using PCR Clean-Up & Gel Extraction Kit (GeneDirex, USA). Briefly, the nucleotide sequences of 16s rRNA gene were carried out by next generation sequencing (Macrogen, Seoul, Korea). The taxonomy of nucleotide sequences was analyzed to compare with a homology species by bacterial 16s rRNA gene database using BLAST search and EzTaxon bioinformatics software. The closest relative species of the 16s rRNA gene sequences were analyzed. A similarity $\geq$ 99% to 16s rRNA gene sequences of type isolate were used as the criterion for identification. Finally, probiotic LAB species with ability to produce BSH which listed in the Food and Drug Administration (FDA) of Thailand were selected for the candidate of cholesterol-lowering probiotic LAB. Finally, they were presented the genotypic relation of 16s rRNA gene sequences as phylogenetic tree with 1000 bootstraps in MEGA 7.0 software. The candidate of cholesterol-lowering probiotic

LAB was used to study in the next experiments according to the FDA guidelines of Thailand, including safety for use, adherence property assay, and acid and bile tolerance assay.

1.6 Qualitative investigation of hemolytic activity using a spot plating technique

Preparation of sheep blood agar, blood was mixed homogeneously for 5 min and then they were warmed at 30 ˚C prior to use for this study. Sheep blood was aseptically added to Brain heart infusion (BHI; HiMedia, India) agar at a final concentration of 5% (v/v) blood. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 24 h. After that, they were adjusted to 10^9 CFU/mL with PBS, pH 7.2 and spotted onto 5% (v/v) sheep blood agar followed by anaerobic incubation at 37 ˚C for 48 h. After incubation, bacterial colony with hemolytic zone was interpreted as a positive result. *Streptococcus pyogenase* DMST 4478 and *E. faecalis* DMST 4737 were used as positive and negative control, respectively.

1.7 Antibiotic susceptibility assay using disk diffusion method

Eight representative antibiotic disks (Thermo Fisher Scientific, USA), including ampicillin (10 ug), chloramphenicol (30 ug), erythromycin (15 ug), gentamycin (10 ug), nalidixic acid (30 ug), penicillin G (10 U), tetracycline (30 ug), and vancomycin (30 ug) were used in this experiment for assessment of antibiotic susceptibility. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 24 h. They were adjusted to 10^8 CFU/mL with PBS, pH 7.2 and swabbed thrice onto Mueller-Hinton (MH; HiMedia, India) agar. After that, antibiotic disks were placed on MHA and anaerobically incubated at 37 ˚C for 24 h. Antibiotic susceptibility were interpreted based on the diameter of inhibition zone. Blank disk was used as control.

1.8 Investigation of gamma-amino butyric acid biosynthesis using thin layer chromatography

Glutamic acid and monosodium glutamate (Sigma-Aldrich, USA) were selected as a substrate to determine the gamma-amino butyric acid (GABA) production. MRS broth was supplemented with 5% (w/v) either glutamic acid or monosodium glutamate. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ and adjusted to 10^9 CFU/mL with MRS broth supplemented with either glutamic acid or monosodium glutamate followed by anaerobic incubation at 37 ˚C for 24 h. After incubation, bacterial culture was centrifuged at 5,000 g, 25 ˚C for 10 min to harvest the supernatant. Then, 0.2 uL of supernatant was spotted onto thin layer chromatography (TLC) Silica gel 60 F_{254} plates (Merck, Germany) and then TLC plates was placed in the TLC chamber with mobile phase (ingredients: 50% (v/v) n-butanol, 30% (v/v) acetic acid and 20% (v/v) distilled water) until they closely reach to the edge of TLC plates. Separated spot of GABA was compared with standard GABA (Sigma-Aldrich, USA). *Lactobacillus brevis* and MRS broth supplemented with either glutamic acid or monosodium glutamate was used as positive control and blank, respectively.(106)

1.9 Growth characteristic analysis

Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ and sub-cultured into MRS broth followed by anaerobic incubation at 37 °C for 24 h. After that, they were adjusted to 10^9 CFU/mL with MRS broth and inoculated in MRS broth at a final concentration of 0.1% (v/v) bacterial cells, then they were anaerobically incubated at 37 ˚C. The optical absorbance at 600 nm of MRS broth during incubation was continuously every 2 h until they reach to stationary phase.

1.10 Antimicrobial activity assay using modified agar well diffusion method

Indicator strains of pathogen, including *S. aureus* DMST 20654, *S. pyogenase* DMST 4478, *Proteus mirabilis* ATCC 13315, *Shigella dysenteriae* DMST 15111, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 21853, *Vibrio cholerae* DMST 2873, *V. parahaemolyticus* DMST 5665, *Salmonella typhi* DMST 5784, *B. subtilis* ATCC 6633, *Klebsiella pneumonia* DMST 8216, and *S. pneumoniae* DMST 48997 were grown in nutrient agar (HiMedia, India) under aerobic incubation at 37 ˚C for 24 h. After incubation, they were adjusted to 10^8 CFU/mL with PBS, pH 7.2 and swabbed thrice onto MHA followed by drilling the well. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 24 h and then they were adjusted to 10^9 CFU/mL with MRS broth followed by anaerobic incubation at 37 ˚C for 24 h. After incubation, bacterial culture was centrifuged at 5,000 g, 25 ˚C for 10 min to harvest the supernatant. Forty-five uL of supernatant was added into the well of MHA plates and aerobically incubated at 37 ˚C for 24 h. MRS broth was used as blank control. For reuterin detection, Cholesterol-lowering probiotic LAB were adjusted to 10^9 CFU/mL with 2% (v/v) glycerol followed by anaerobic incubation at 37 ˚C for 24 h. Antimicrobial activity of reuterin was performed as described above.

Antimicrobial activity was interpreted based on diameter of inhibition zone: strong (diameter ≥ 20.1 mm), moderate (10.1 mm ≤ diameter ≤ 20.0 mm), weak (4.1 mm ≤ diameter ≤ 10.0 mm) and no zone (diameter ≤ 4.0 mm).(107)

1.11 Adherence property assay

1.11.1 Caco-2 cell culture

Human colon adenocarcinoma cell line (Caco-2; ATCC, HTB-37) was used as a model of colon epithelial cells for assessment of probiotic adhesion. Initially, Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Waltham, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Waltham, USA) and 1% (v/v) Penicillin-Streptomycin (Gibco, Waltham, USA) followed by incubation at 37 ˚C in a 5% CO_2 atmosphere. In the meantime, culture medium was replaced alternate day until at least 70% confluent. After that, Caco-2 cells monolayer was trypsinized and seeded in 24-well tissue culture plate (Corning, USA) at a concentration 1×10^5 cells/mL followed by incubation at 37 ˚C in a 5% CO_2 atmosphere for 15 days. Then, old culture medium was replaced with 2 mL of antibiotic free medium prior the adherence assay.

1.11.2 *In vitro* adherence assay

Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ and sub-cultured into MRS broth. They were adjusted to 10^9 CFU/mL and washed twice with PBS, pH 7.2 by centrifugation at 5,000 g, 25 ˚C for 10 min. Bacterial cells pellet was re-suspend with antibiotic free medium and added to 24-well tissue culture plate with Caco-2 cells monolayer (test) or without Caco-2 cells monolayer (control). *L. rhamnosus* GG (LMG 18243) was used as reference strain which was carried out along with the other strains. After that, 24-well tissue culture plate was incubated at 37 ˚C in a 5% CO_2 atmosphere for 1 h and then each well was washed thrice with PBS, pH 7.2 to remain only adhered bacterial cells. After that, Caco-2 cells were lysed by 0.5% (v/v) Triton® X-100 (Merck, Germany) to harvest probiotic LAB cells. For bacterial cells count, bacterial suspension was serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h.(108) Finally, the number of colonies was analyzed as the exact Log_{10} CFU/mL.

1.12 Acid and bile tolerance assay

Cholesterol-lowering probiotic LAB were evaluated in this experiment for assessment the relative survivability in the gastrointestinal conditions.

1.12.1 Acid tolerance assay

Preparation of acid condition, MRS broth was adjusted with 1M of hydrochloric acid (HCl; Merck, Germany) to pH 2.0, 3.0, and 4.0, respectively. Unadjusted MRS broth was used as normal control. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ and sub-cultured into MRS broth. After that, they were inoculated in each acid condition at a final concentration of 10^9 CFU/mL followed by anaerobic incubation at 37 ˚C for 3 h. After incubation, acid treated bacterial cells were serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h. The number of colonies was analyzed as the exact Log_{10} CFU/mL of viable cells.

1.12.2 Bile tolerance assay

Preparation of bile condition, MRS broth was adjusted to 0.3 and 0.8% (w/v) bovine bile (Sigma-Aldrich, USA), respectively. Unadjusted MRS broth was used as normal control. Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ and sub-cultured into MRS broth. After that, they were inoculated in each bile condition at a final concentration of 10^9 CFU/mL followed by anaerobic incubation at 37 ˚C for 3 h. After incubation, bile treated bacterial cells were serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h. The number of colonies was analyzed as the exact Log_{10} CFU/mL of viable cells. *L. rhamnosus* GG was used as reference strain.(109)

2 Determination of cholesterol-lowering effect of candidate probiotic lactic acid bacteria on high cholesterol diet-induced rat model

Cholesterol-lowering probiotic LAB were used in this experiment for confirmation the effect of cholesterol reduction on high cholesterol diet-induced rat model. The ethic approval number was COA / AE-013-2562.

2.1 Animal housing and feeding

Thirty-five male wistar rats (*Rattus norvegicus*) aged 8 weeks with an average weight between 120 and 140 g which purchased from the Nomura Siam International (Bangkok, Thailand) were used in this experiment. The rats were housed in plastic cage with wire mesh clover in a room condition at a temperature of 24±1 ˚C, a light-dark cycle of 12/12 h and 60±5% relative humidity.

Prior commencement of the experiment, the rats were acclimatized for 1 week with *ad libitum* food (082G/15 basal diet) and water. After acclimation period, the rats were collected feces (pre experimental procedure) to determine gut microbiota and then they were randomly assigned into 7 experimental groups with 5 rats in each group as follow: (1) NC, high cholesterol free diet control; (2) HF, high cholesterol enriched diet control; (3) NCP, high cholesterol free diet with probiotic isolates 1,2 and 3; (4) HFP1, high cholesterol enriched diet with probiotic isolate 1; (5) HFP2, high cholesterol enriched diet with probiotic isolate 2; (6) HFP3, high cholesterol enriched diet with probiotic isolate 3; and (7) HFP123, high cholesterol enriched diet with probiotic isolates 1, 2 and 3. For supplementation, the other 5 groups without NC and NCP group were administered by daily oral gavage with 1 mL of high cholesterol enriched diet, while the NCP, HFP1, HFP2, HFP3 and HFP123 group were administered by daily oral gavage with 1 mL of probiotics suspended with PBS, pH 7.2 at a concentration of 10^9 CFU/mL as designed above. Finally, HF group was administered by daily oral gavage with 1 mL of PBS, pH 7.2, and then all groups were received with *ad libitum* food and water according to summarize in Table 4.

Probiotic LAB isolates 1, 2, and 3 were represented as *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* subsp. *lactis* TA-1, respectively.

This experiment was performed for 8 weeks. During experimental period, the body weight of rat was recorded every 2 weeks, while feces were collected pre and post experimental procedure for analysis of gut microbiota. At the end of experimental period, the rats were fasted for 12 h and euthanized using isofurane (Piramal clinical care, USA). After euthanasia, blood samples (post experimental procedure) were collected by cardiac puncture and preserved in heparinized vacuum tube. All specimens of rat were used to study in the next experiments.(110)

Table 4 Diet information of seven experimental groups

Code group	Information group	Diet with supplement
NC	High cholesterol free diet (negative control)	Basal diet + 2 mL PBS, pH 7.2
HF	High cholesterol enriched diet (positive control)	Basal diet + 1 mL high cholesterol enriched diet + 1 mL PBS, pH 7.2
NCP	High cholesterol free diet with probiotic isolates 1,2 and 3	Basal diet + 1 mL probiotic isolates 1, 2 and 3 + 1 mL PBS, pH 7.2
HFP1	High cholesterol enriched diet with probiotic isolate 1	Basal diet + 1 mL high cholesterol enriched diet + 1 mL probiotic isolate 1
HFP2	High cholesterol enriched diet with probiotic isolate 2	Basal diet + 1 mL high cholesterol enriched diet + 1 mL probiotic isolate 2
HFP3	High cholesterol enriched diet with probiotic isolate 3	Basal diet + 1 mL high cholesterol enriched diet + 1 mL probiotic isolate 3
HFP123	High cholesterol enriched diet with probiotic isolates 1, 2 and 3	Basal diet + 1 mL high cholesterol enriched diet + 1 mL probiotic isolates 1, 2 and 3

Table 5 Nutritional composition of basal diet (082G/15)

Basal diet (082G/15)	% by weight
Moisture (max)	12.6
Crude protein (min)	24
Fat (min)	4.5
Fiber (max)	5
Vitamins mix	-
Mineral salts	-

Metabolizable energy = 2,040 (Kcal/kg)

Table 6 Nutritional composition of high cholesterol enriched diet

High cholesterol enriched diet	% by weight
Beef tallow	17.2
Margarine or butter	20
Egg yolk	45
Sucrose	12.5
Cholesterol powder	1.25
Sodium cholate	0.5

Metabolizable energy = 4,925 (Kcal/kg)

2.2 Biochemical analysis

To analyze the plasma lipid profiles, heparinized blood was immediately centrifuged at 1,500 g, 25 ˚C for 15 min to separate plasma from blood cells. Subsequently, the plasma was determined lipid profiles, including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) by using Qucare® Multi Meter kits (DFI care, Gyeonggido, South Korea).(111)

2.3 Gut microbiota analysis

To determine the amount of microorganisms in the intestine of rats. The feces of rat were dispersed and serially diluted 10-fold with PBS, pH 7.2. Each dilution was spread on selective media, including MRS agar with $CaCO_3$, BSM agar with *Bifidobacterium* selective supplement, and Eosin Methylene Blue (EMB; HiMedia, India) agar followed by anaerobic incubation at 37 ˚C for 48 h. After incubation, the colony was stained by gram stain and the number of colony was counted with distinctive morphology. Finally, the amount of each microorganism in the intestine of rats was presented as the Log_{10} CFU/g feces.(112)

3 Application of cholesterol-lowering probiotic lactic acid bacteria via microencapsulation

Cholesterol-lowering probiotic LAB were used in this experiment for development of cholesterol-lowering probiotic products via microencapsulation.

3.1 Cholesterol-lowering probiotic lactic acid bacteria culture conditions

Cholesterol-lowering probiotic LAB were grown in MRS agar with $CaCO_3$ under anaerobic incubation at 37 ˚C for 48 h. After incubation, they were adjusted to 10^9 CFU/mL with MRS broth and anaerobically incubated at 37 ˚C until they reached to early stationary phase. After that, they were adjusted to 10^{10} CFU/mL and washed thrice with PBS, pH 7.2 by centrifugation at 7,000 g, 4 ˚C for 10 min following by re-suspension with PBS, pH 7.2 for experimental use. Bacterial cells suspension was divided to dilute at 10^9 CFU/mL with PBS, pH 7.2 and separately stored at 4 and 25 ˚C for up to 12 weeks in order to use as long term storage model of free cells.

3.2 Preparation of nano-crystalline starch from *Manihot esculenta* Crantz

Cassava starch (Siam Quality Starch, Thailand) was prepared at a concentration of 20% (w/v) with 3.20 M of H_2SO_4 (Merck, Germany) followed by shaking incubation at 120 rpm, 37 ˚C for 170 h in order to hydrolyze starch granules. After incubation, sample was taken for cool down at room temperature for 30 min and filtered with 0.45 um membrane filter (Merck, Germany) to separate starch granules. After that, cassava starch was rinsed with deionized water to wash the acidity until the pH value reached to 7 and then sample was dispersed in deionized water and homogenized at 12,000 rpm for 5 min. For drying, the cassava starch solution was added to round bottom flask and pre froze at -40 ˚C for 10 min in the ethanol bath. After pre-freezing, sample was freeze dried by Freeze dryer (SciQuip, UK) for overnight. Then, nano-crystalline starch (NCS) was collected in sterile bottles and stored at 25 ˚C in desiccator (Thomas Scientific, USA).(113)

3.3 Nano-crystalline starch analysis

Structure of NCS was analyzed using X-ray diffractometer (Bruker, Germany). Briefly, the copper (CuK_α) of X-ray source was adjusted wavelength at 1.5406 angstrom and velocity of 2-Theta value was 0.02 ˚2-Theta/second. Sample was scanned with X-ray, then X-ray diffractogram was analyzed and represented as crystallinity with OriginPro Data Analysis and Graphing Software. Finally, relative crystallinity was calculated and presented as the percentage following to the equation:

$$\text{Relative crystallinity (\%)} = \left(\frac{\text{crystalline area}}{\text{crystalline area + amorphous area}}\right) \times 100$$

3.4 Microencapsulation processes by whey protein isolate and nano-crystalline starch

To develop cholesterol-lowering probiotic products via spray drying microencapsulation. Whey protein isolate (WPI) and NCS were separately prepared as described below. Briefly, 35 g (WPI formula) or 28 g (WPI-NCS formula) of WPI solution was prepared in 500 mL of sterile deionized water and then solution was stirred at 250 rpm for 30 min in order to dissolve completely. After that, 7 g of nano-crystalline starch was added in WPI-NCS solution immediately (7% total solid), and stored at 4 ˚C for 12 h. The solution was adjusted pH to 7.5 with 5 M of sodium hydroxide (NaOH) followed by heating at 90 ˚C for 10 min to denature protein. The mixture was added by 0.1% (w/v) gum arabic and homogenized at 12,000 rpm for 10 min to make encapsulating matrix at ratio of 4:1 (WPI-NCS formula) and WPI alone (WPI formula). Then, cholesterol-lowering probiotic LAB were added into encapsulating matrix followed by stirrer at 200 rpm for 5 min. For spray drying, the mixture was added to chamber stirrer for microencapsulation using a Mini Spray Dryer B-290 (Buchi, Switzerland) with an air inlet temperature of 120 ˚C and air outlet temperature of 75 ˚C, spray flow feed rate of 0.27 L/h. Finally, the microcapsules were collected from cyclone chamber. They were stored in the sterile bottles at 4 and 25 ˚C. (114) The significant information is the confidentiality in the patent (patent application number 2001002960).

3.5 Morphological analysis using scanning electron microscope

The morphology of microcapsule was analyzed using scanning electron microscope (Hitachi High-Technologies, Japan). Sample was prepared on stub using carbon tape and coated with gold particle to electrical conduction. The condition of electron under the high vacuum was set at electric potential acceleration of 15.0 kV.

3.6 Microencapsulation yield

Encapsulated cholesterol-lowering probiotic LAB were used in this experiment in order to measure viability of probiotic cells after spray drying. The encapsulated cholesterol-lowering probiotic LAB were released with PBS, pH 7.2 and then they were serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h. Finally, the number of colonies was analyzed as the percentage of yield.(115)

3.7 Survivability of free and encapsulated cholesterol-lowering probiotic lactic acid bacteria after sequential incubation in the simulated gastrointestinal conditions

The simulated gastric juice and simulated intestinal juice were used as a gastrointestinal condition models to study the survival of probiotics in the microcapsule products after exposure the gastrointestinal conditions. Initially, the simulated gastric juice (SGJ) was prepared using 0.2% (w/v) sodium chloride (NaCl) solution, including 0.35% (w/v) pepsin (Sigma-Aldrich, USA) and then pH was adjusted to 2.0 with 1M of HCl. The simulated intestinal juice (SIJ) was prepared using 0.2% (w/v) NaCl solution, including 0.1% (w/v) trypsin (Sigma-Aldrich, USA), 1.0% (w/v) bovine bile, and 1.1% (w/v) sodium bicarbonate ($NaHCO_3$) and then pH was adjusted to 8.0 with 1M of NaOH. Finally, these solutions were sterilized by 0.22 um membrane filter.(116)

One gram of free and encapsulated cholesterol-lowering probiotic LAB were added to SGJ and followed by anaerobic incubation at 37 ˚C for 3 h. After that, cholesterol-lowering probiotic LAB were harvested by centrifugation at 5,000 g, 4 ˚C for 10 min. Pellet was added into SIJ and followed by anaerobic incubation at 37 ˚C for 3 h. One hour interval of incubation, 1 mL of the simulated juices was harvested and then they were serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h. The number of colonies was analyzed as the exact Log_{10} CFU/g of viable cells.(117)

3.8 Stability of free and encapsulated cholesterol-lowering probiotic lactic acid bacteria during long term storage

To study the stability of encapsulated cholesterol-lowering probiotic LAB during storage of microcapsule products. Free and encapsulated cholesterol-lowering probiotic LAB were stored at 4 and 25 ˚C for 12 weeks. Every week, 1 g of free and encapsulated cholesterol-lowering probiotic LAB were evaluated the stability during storage. Briefly, 1 g of sample was suspended in 10 mL of PBS, pH 7.2 and then they were serially diluted 10-fold with PBS, pH 7.2 and spread on MRS agar with $CaCO_3$ followed by anaerobic incubation at 37 ˚C for 48 h. The number of colonies was analyzed as the exact Log_{10} CFU/g of viable cells in each week.(118)

4 Statistical analysis

All experiments were performed three replicates independently. The results were expressed as means ± standard deviation. The independent t-test and analysis of variance (ANOVA) with Dunnett's or Tukey's multiple comparison test ($p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$) were performed to analyze the statistically significant of result by GraphPad Prism Software Version 8.0 (San Diego, CA, USA).

CHAPTER 4

RESULTS

1 Functional characterization of probiotic lactic acid bacteria with bile salt hydrolase activity

1.1 Isolation and selection of probiotic lactic acid bacteria

A total of 124 probiotic lactic acid bacteria (LAB) isolates was found in 50 traditional food samples. However, only 74 probiotic LAB isolates with distinct morphology showed the desired characteristics, including gram-positive, catalase-negative, and lactic acid production. All 74 probiotic LAB isolates were stored at -80 ˚C for experimental use.

1.2 Motility and cholesterol-lowering activity of probiotic lactic acid bacteria

These probiotic LAB isolates were tested the ability to motile, which it indicates the virulence factor from cilia or flagella. The result of these probiotic LAB isolates was non-motile which indicate that they were not pathogenic bacteria. Thus, they were approved to determine cholesterol-lowering activity. Bile salt hydrolase (BSH) activity is the vital ability of cholesterol reduction, from the result in Table 7 showed BSH activity on both taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA) with different ability in only 17 probiotic LAB isolates (Figure 12A and B). TF-7 and TF-18 isolate showed the strongest BSH activity both TDCA and GDCA when compared with the other isolates. The assimilation of cholesterol was tested to confirm the cholesterol-lowering activity, they exhibited range from 14.00±1.13% to 58.97±1.60%. TF-7 and TF-18 isolate also exhibited cholesterol assimilation of 58.97±1.60% and 57.98±4.35%, respectively. However, 17 probiotic LAB isolates were further confirmed their probiotic species by 16S ribosomal RNA (rRNA) gene sequencing.

Table 7 Cholesterol-lowering activity of probiotic lactic acid bacteria isolates

Isolates	Source	Cholesterol-lowering activity		
		BSH activity[a]		Cholesterol assimilation (%)
		TDCA	GDCA	
TF-1	Dried mango paste	++	+	25.24±1.70
TF-3	Pickled salak	-	+	21.88±1.27
TF-5	Pickled mustard	+	++	14.91±1.63
TF-6	Pickled bamboo shoot	+	+	43.96±4.39
TF-7	Pickled olives	+++	++	58.97±1.60
TF-9	Raw pork meat	+++	-	33.42±3.58
TF-12	Salted plum	++	+	17.62±5.80
TF-14	Agasta	++	-	41.89±3.14
TF-16	Pickled garlic	+	++	26.76±2.53
TF-17	Pickled santol	++	+	32.24±2.51
TF-18	Sweet fermented rice	+++	+++	57.98±4.35
TA-1	Raw cow milk	+	++	36.91±2.01
TA-2	Sticky rice	+	+	40.83±3.98
TA-6	Pickled grape	-	++	18.89±5.24
TA-7	Sticky rice	++	+	30.25±2.10
TA-10	Pickled bamboo shoot	+	-	14.00±1.13
TA-12	Pickled crab	+	++	18.80±3.44

[a] BSH activity was interpreted based on the diameter of precipitation zone (mm): -, no zone; +, ≤ 8.0 mm (weak); ++, 8.1 mm–13.0 mm (moderate); +++, ≥ 13.1 mm (strong). (n=3, mean±SD)

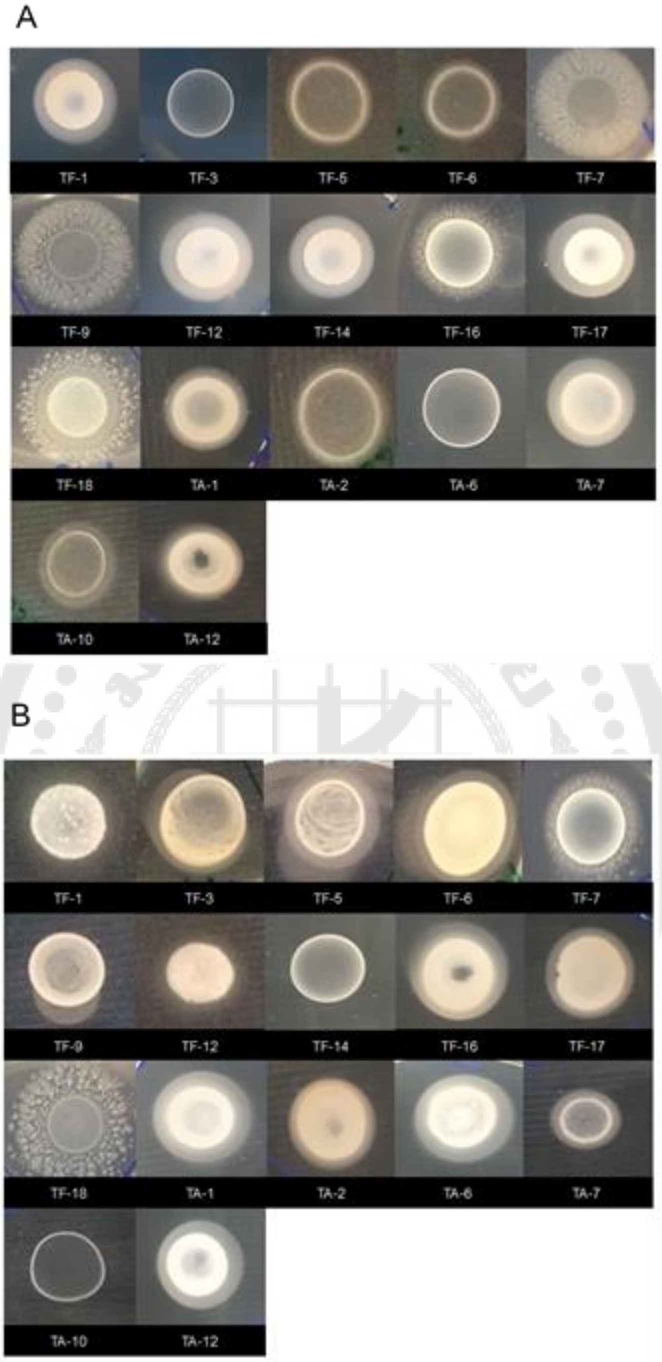


Figure 12 Bile salt hydrolase activity of probiotic lactic acid bacteria isolates

BSH activity on MRS agar supplemented with (A) 0.5% (w/v) TDCA and (B) 0.5% (w/v) GDCA.

1.3 Genotypic identification (16s rRNA) of selected probiotic lactic acid bacteria

Seventeen probiotic LAB isolates with cholesterol-lowering activity were identified with their 16S rRNA gene to verify species-level of probiotic LAB, compared with type strains in NCBI GenBank database using BLAST-search and EzTaxon bioinformatics software. From the result in Table 8 showed species and strains of 17 probiotic LAB isolates, they belonged to 4 genera, including *Lactobacillus* (71%), *Streptococcus* (6%), *Enterococcus* (17%), and *Bifidobacterium* (6%). Only different genera of LAB isolates, which extensively possessed cholesterol-lowering activities, based on the most robust BSH activity and cholesterol assimilation comparing with their same genera and list of the guidelines of Food and Drug Administration (FDA) of Thailand, were selected as the candidates of cholesterol-lowering LAB for further study. Therefore, TF-7, TF-18, and TA-1 isolate were selected for the candidates of cholesterol-lowering LAB defined following the type strains in NCBI GenBank database with strains *L. reuteri* JCM 1112, *E. faecium* LMG 11423, and *B. animalis* subsp. *lactis* DSM 10140 under the strain names as *L. reuteri* TF-7 (99.79% identity), *E. faecium* TF-18 (99.51% identity), and *B. animalis* subsp. *lactis* TA-1 (99.57% identity), respectively. Finally, the genotypic relation of 16S rRNA gene-based nucleotide sequences of cholesterol-lowering LAB was presented as phylogeny among closely related species of the genus *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* using MEGA program (version 7.0) with 1000 neighbor-joining method of bootstrap analysis (Figure 13). These three LAB strains are major genera of probiotics, leading to the diversity of gut microbiota. Although, *B. animalis* subsp. *lactis* TA-1 had weak to moderate BSH activity, and moderate cholesterol assimilation, it was selected as cholesterol-lowering LAB strain because it was the only one isolate identified as *Bifidobacterium* genus among the total of 17 LAB isolates.

Table 8 Genotypic identification (16s rRNA) of probiotic lactic acid bacteria isolates

Isolates	Species and strains	Identity (%)
TF-1	*Lactobacillus mucosae* S32	99.09
TF-3	*Streptococcus gallolyticus* subsp. *macedonicus* NCTC 13767	99.51
TF-5	*Lactobacillus fermentum* E4	99.79
TF-6	*Lactobacillus fermentum* E1	100.00
TF-7	*Lactobacillus reuteri* JCM 1112	99.79
TF-9	*Lactobacillus mucosae* S32	99.74
TF-12	*Enterococcus durans* NBRC 100479	99.64
TF-14	*Lactobacillus agilis* DSM 20509	99.44
TF-16	*Lactobacillus fermentum* C8.1	99.66
TF-17	*Lactobacillus animalis* KCTC 3501	99.65
TF-18	*Enterococcus faecium* LMG 11423	99.51
TA-1	*Bifidobacterium animalis* subsp. *lactis* DSM 10140	99.57
TA-2	*Lactobacillus gasseri* DMBCT6	99.51
TA-6	*Lactobacillus reuteri* LGM7-1	99.35
TA-7	*Lactobacillus johnsonii* ATCC 33200	99.79
TA-10	*Lactobacillus amylovorus* DSM 20531	84.20
TA-12	*Enterococcus faecium* LAC7.2	99.64

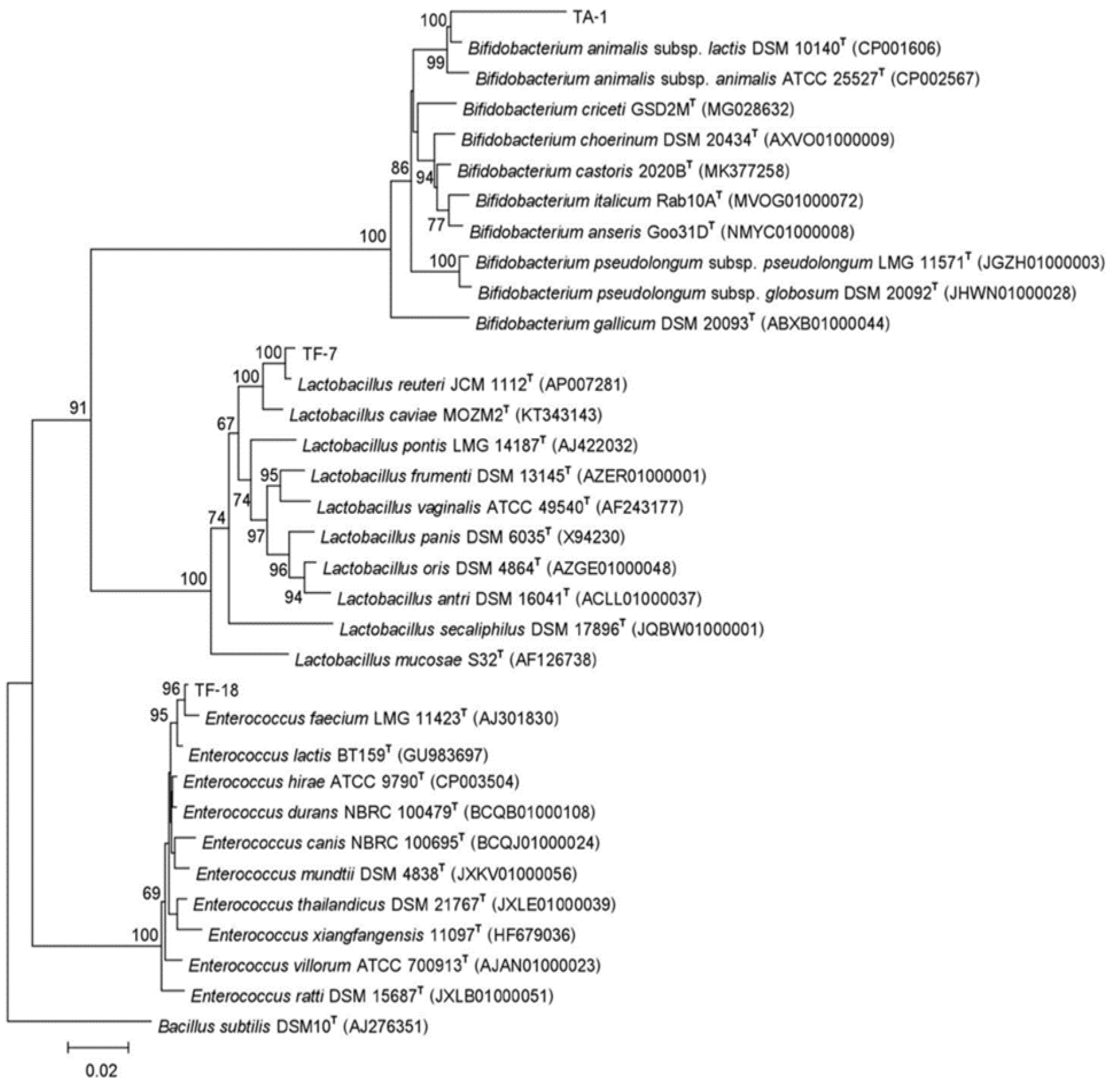


Figure 13 Phylogeny of cholesterol-lowering probiotic lactic acid bacteria

Phylogeny was created by neighbour-joining method with 1000 bootstrap values.

1.4 Safety evaluation of cholesterol-lowering probiotic lactic acid bacteria

To confirm the safety of cholesterol-lowering probiotic LAB for development as probiotic products according to the FDA guidelines of Thailand.(119) The result in Figure 14 displayed that three isolates of cholesterol-lowering probiotic LAB exhibited non-hemolysis when compared with positive and negative control, respectively, which indicate that three isolates did not have a hemolytic toxin. Next, the antibiotic susceptibility was performed to evaluate the resistant ability of cholesterol-lowering probiotic LAB (Table 9). All three isolates were susceptible to chloramphenicol, nalidixic acid, erythromycin, and tetracycline. Anywise, penicillin was highly resistant rate, as all three isolates were resistant to penicillin. TF-18 and TA-1 isolates were resistant to vancomycin, while only TF-18 isolate was semi-susceptible to ampicillin and gentamicin. TF-7 isolate was also semi-susceptible to gentamicin. Thus, TF-18 and TA-1 isolates were more resistant than TF-7 isolate with resistant rates of 25, 25, and 12.5%, respectively.

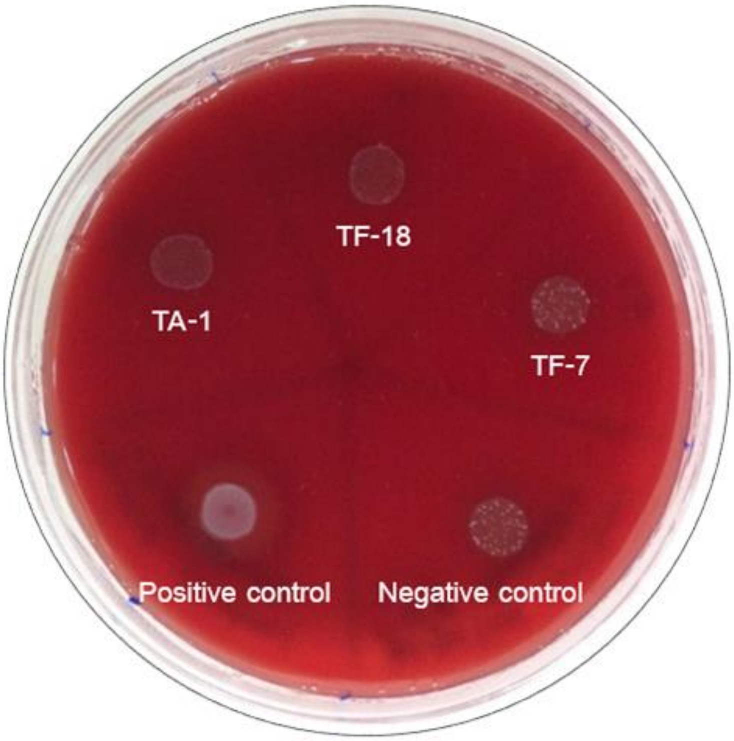


Figure 14 Hemolytic activity of cholesterol-lowering probiotic lactic acid bacteria

Hemolytic activity of cholesterol-lowering probiotic LAB were compared with positive and negative control, respectively.

Table 9 Antibiotic susceptibility of cholesterol-lowering probiotic lactic acid bacteria

Antibiotics	Antibiotic susceptibility (mm)		
	TF-7	TF-18	TA-1
Ampicillin	25.67±0.58 (S)	16.00±1.73 (I)	22.67±0.58 (S)
Chloramphenicol	31.67±0.58 (S)	34.00±1.00 (S)	26.33±0.58 (S)
Gentamicin	8.33±0.58 (I)	8.67±0.58 (I)	30.33±0.58 (S)
Nalidixic acid	30.67±1.15 (S)	31.67±0.58 (S)	31.33±1.15 (S)
Penicillin	11.33±1.15 (R)	7.67±1.15 (R)	9.00±1.73 (R)
Erythromycin	25.33±0.58 (S)	26.33±0.58 (S)	26.00±1.00 (S)
Tetracycline	31.00±1.00 (S)	34.67±0.58 (S)	29.33±1.15 (S)
Vancomycin	21.67±2.08 (S)	0.00±0.00 (R)	8.33±1.53 (R)

Antibiotic susceptibility was interpreted according to the diameter of inhibition zone (mm), antibiotic disk with a diameter of 6.5 mm. (n=3, mean±SD)

GEN: (R), ≤ 8.0 mm (resistant); (I), 8.1 mm–10.0 mm (intermediate); (S), ≥ 10.1 mm (susceptible)

ERY: (R), ≤ 13.0 mm (resistant); (I), 13.1 mm–23.0 mm (intermediate); (S), ≥ 23.1 mm (susceptible)

VAN: (R), ≤ 12.0 mm (resistant); (I), 12.1 mm–13.0 mm (intermediate); (S), ≥ 13.1 mm (susceptible)

Other antibiotics: (R), ≤ 12.4 mm (resistant); (I), 12.5 mm–17.4 mm (intermediate); (S), ≥17.5 mm (susceptible)

1.5 Gamma-amino butyric acid biosynthesis of cholesterol-lowering probiotic lactic acid bacteria

The determination of gamma-amino butyric acid (GABA) biosynthesis was performed to determine GABA production using thin layer chromatography (TLC). Glutamic acid and monosodium glutamate were used as the substrate to produce GABA by glutamic acid decarboxylase (GAD). The result in Figure 15A and B showed that only TF-7 isolate was able to produce GABA from both glutamic acid and monosodium glutamate when compared with GABA standard, positive, and negative control, respectively.

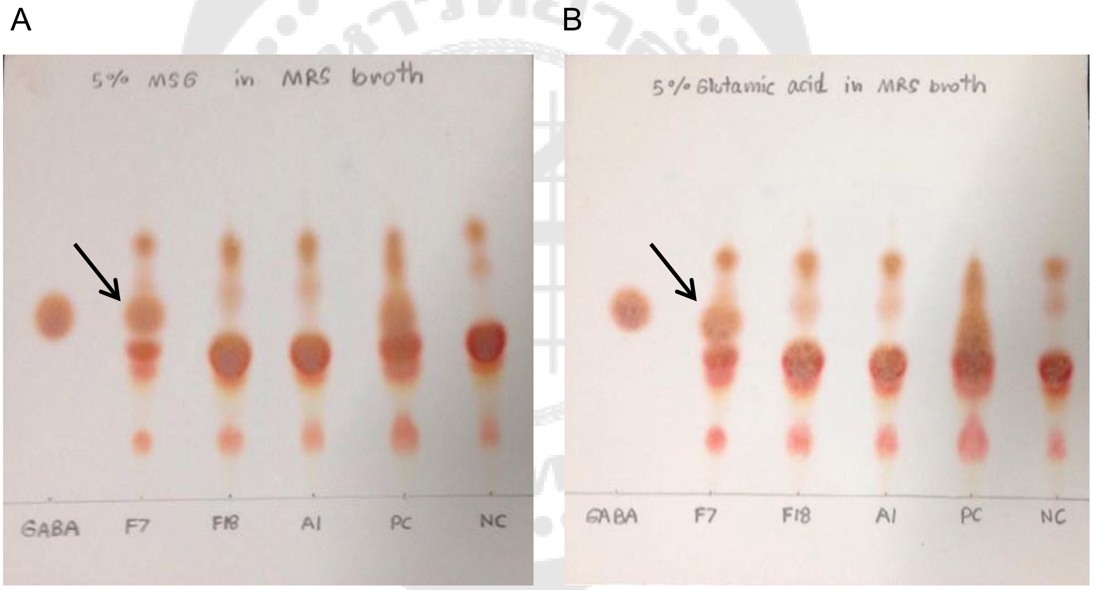


Figure 15 Gamma-amino butyric acid biosynthesis of cholesterol-lowering probiotic lactic acid bacteria

GABA production in MRS broth supplemented with (A) 5% (w/v) monosodium glutamate and (B) 5% (w/v) glutamic acid, compared with GABA standard, positive, and negative control, respectively.

1.6 Growth characteristics of cholesterol-lowering probiotic lactic acid bacteria

Three cholesterol-lowering probiotic LAB were determine the growth characteristics, because their growth phase were used to apply in the culture conditions for development of probiotic products. From the result in Figure 16 revealed that early stationary phase of TF-7, TF-18, and TA-1 isolate were 18, 16, and 56 h, respectively.

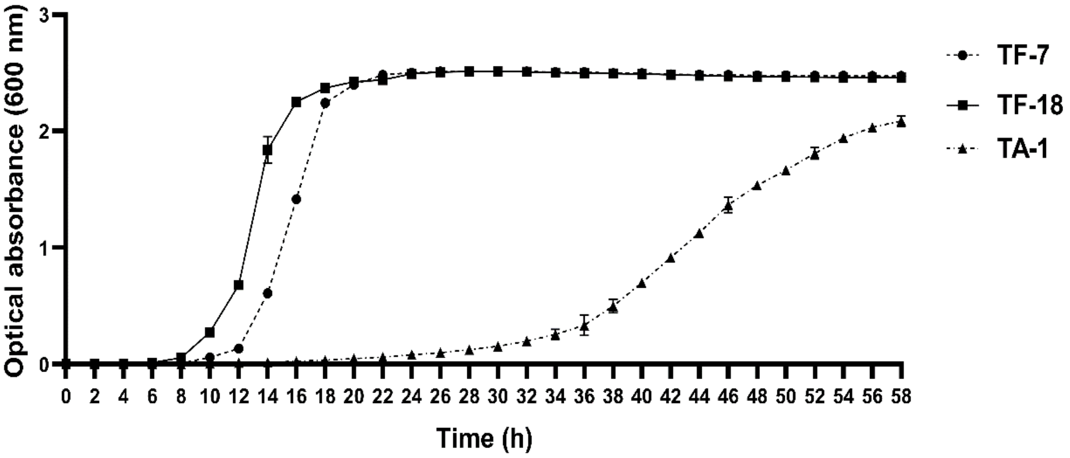


Figure 16 Growth characteristics of cholesterol-lowering probiotic lactic acid bacteria

The optical absorbance values were represented as bacterial growth. (n=3, mean±SD)

1.7 Antimicrobial activity of cholesterol-lowering probiotic lactic acid bacteria

Antimicrobial activity of cholesterol-lowering probiotic LAB was tested against 12 clinical pathogens as shown in Table 10. Three isolates were expressed antagonistic activity against different pathogen, TF-7 isolate was exhibited broad-spectrum inhibition with moderate activity against *S. pyogenase* and weak activity against *S. aureus*, *P. mirabilis*, *V. cholerae*, and *V. parahaemolyticus*. For reuterin of TF-7 isolate showed strong and moderate antagonistic activity against *S. pyogenase* and *S. aureus*, respectively. While TF-18 isolate was the slightest activity, it could inhibit only

P. mirabilis. TA-1 isolate was inhibited the growth of *P. mirabilis* and *V. parahaemolyticus* with weak activity. Thus, TF-7 isolate exhibited the strongest antagonistic activity with rate of 42%, while TF-18 and TA-1 isolate had the antagonistic rates of 8 and 17%, respectively.

Table 10 Antimicrobial activity of cholesterol-lowering probiotic lactic acid bacteria

Pathogens	Antimicrobial activity (mm)			
	TF-7	TF-18	TA-1	TF-7 reuterin
S. aureus	7.00±1.73 (W)	0.00±0.00 (N)	0.00±0.00 (N)	12.33±0.58 (M)
S. pyogenase	15.67±0.58 (M)	0.00±0.00 (N)	0.00±0.00 (N)	21.67±.0.58 (S)
P. mirabilis	7.33±1.53 (W)	7.00±1.73 (W)	6.67±0.58 (W)	0.00±0.00 (N)
S. dysenteriae	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
E. coli	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
P. aeruginosa	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
V. cholerae	6.67±0.58 (W)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
V. parahaemolyticus	7.00±1.73 (W)	0.00±0.00 (N)	6.67±2.08 (W)	0.00±0.00 (N)
S. typhi	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
B. subtilis	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
K. pneumonia	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
S. pneumoniae	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)

Antimicrobial activity was interpreted according to the diameter of inhibition zone (mm): (N), no zone; (W), ≤ 10.0 mm (weak); (M), 10.1 mm–20.0 mm (moderate); (S), ≥ 20.1 mm (strong), diameter of well was designed at 4.0 mm. (n=3, mean±SD)

1.8 Adherence property of cholesterol-lowering probiotic lactic acid bacteria

To prove the establishment ability in the intestine after administration of cholesterol-lowering probiotic LAB, as an adherence property plays an essential role in the colonization of gut microbiota. Adherence property of three isolates significantly decreased (**** $p < 0.0001$) when compared with their initial cells (Figure 17). However, TF-7 and TF-18 isolates expressed the adherence ability to Caco-2 cells more than *L. rhamnosus* GG, while TA-1 isolate was the lowest adherence ability (Table 11).

Table 11 Adherence property of cholesterol-lowering probiotic lactic acid bacteria

Probiotic cells	Adherence property (Log_{10} CFU/mL)			
	TF-7	TF-18	TA-1	*L. rhamnosus* GG
Initial cells	9.07±0.18	8.97±0.05	9.04±0.06	8.90±0.21
Adhered cells	8.16±0.04	7.33±0.07	6.53±0.21	7.08±0.17

The amount of viable cells was presented as mean±SD. (n=3)

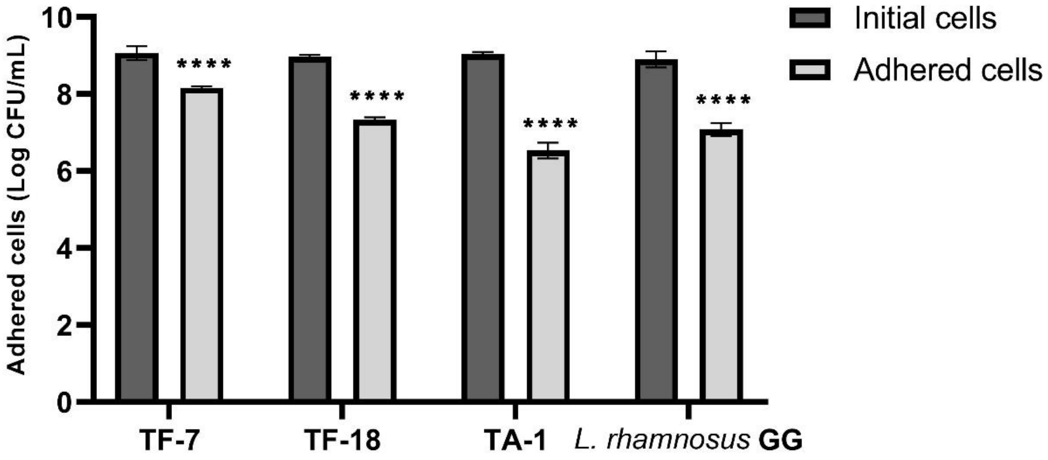


Figure 17 Adherence property of cholesterol-lowering probiotic lactic acid bacteria

Statistical significance of adhered cells was compared with initial cells (**** $p < 0.0001$). (n=3, mean±SD)

1.9 Acid and bile tolerance

When administered probiotics passage through the gastrointestinal tract, the adherence ability of probiotics is an essential characteristic for the colonization in order to facilitate the modulation of nutrient metabolism and synergize the beneficial health effects. However, the harsh environment in the gastrointestinal tract negatively affects bacterial survivability, especially gastric acidity and bile toxicity. Since gastric acid at pH 2.0 can digest several macromolecules of bacterial cells, while concentration of bile salt is able to disperse phospholipid in the plasma membrane. Gastrointestinal tolerance and adherence to intestinal epithelial cells are critical features of probiotics in the gastrointestinal tract. Therefore, cholesterol-lowering probiotic LAB were evaluated the tolerant ability with gastric acid and bile salt because it is the stress condition that negatively affects to the survival rate of cholesterol-lowering probiotic LAB after passage through the gastrointestinal tract.

The result in Table 12 presented that survivability of three isolates after exposure to acid conditions at pH 3.0 and 2.0 gradually decreased with statistically significant (**** $p < 0.0001$), while survivability at pH 4.0 slightly decreased with not significant when compared with unadjusted MRS control (Figure 18A). Moreover, Table 13 presented bile tolerance of three isolates also gradually decreased at 0.3 and 0.8% (w/v) bile with differently significant (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$), compared with unadjusted MRS control (Figure 18B). The reference strain *L. rhamnosus* GG also expressed the trend of acid and bile tolerance similar to cholesterol-lowering probiotic LAB. Anywise, all isolates were able to tolerate under the most stress conditions with survival rates at least 3.46±0.40 Log_{10} CFU/mL and 7.48±0.04 Log_{10} CFU/mL at pH 2.0 and 0.8% (w/v) bile, respectively.

Table 12 Survivability of cholesterol-lowering probiotic lactic acid bacteria in the acid conditions

Probiotic LAB	Survivability in the acid conditions (Log$_{10}$ CFU/mL)			
	MRS control	pH 2.0	pH 3.0	pH 4.0
TF-7	9.13±0.02	3.85±0.20	7.46±0.15	8.97±0.03
TF-18	9.18±0.01	4.05±0.08	7.85±0.13	9.09±0.01
TA-1	9.02±0.03	3.46±0.40	7.40±0.17	8.95±0.03
L. rhamnosus GG	9.16±0.20	3.58±0.24	7.43±0.12	8.96±0.11

The amount of viable cells was presented as mean±SD. (n=3)

Table 13 Survivability of cholesterol-lowering probiotic lactic acid bacteria in the bile conditions

Probiotic LAB	Survivability in the bile conditions (Log$_{10}$ CFU/mL)		
	MRS control	0.3% (w/v) bile	0.8% (w/v) bile
TF-7	9.14±0.02	8.96±0.02	7.85±0.08
TF-18	9.27±0.02	9.10±0.07	8.15±0.08
TA-1	9.12±0.04	8.80±0.07	7.48±0.04
L. rhamnosus GG	9.25±0.10	8.58±0.06	7.49±0.14

The amount of viable cells was presented as mean±SD. (n=3)

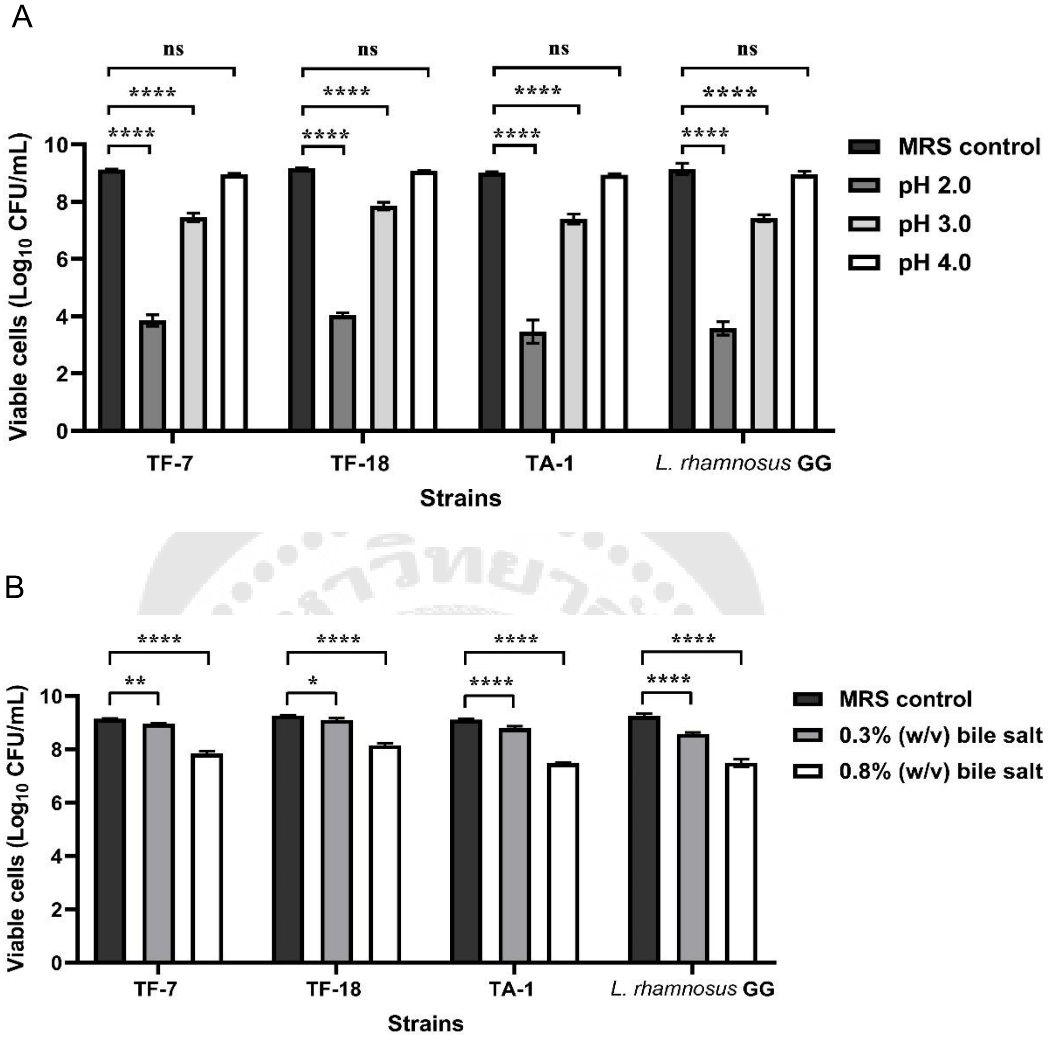


Figure 18 Survivability of cholesterol-lowering probiotic lactic acid bacteria in the acid and bile conditions

(A) Acid conditions and (B) bile conditions, statistical significance of each condition was compared with MRS control (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). (n=3, mean±SD)

The functional characteristics of three cholesterol-lowering probiotic LAB were presented in Table 14. All three strains were exhibited the basically desired properties of probiotics in addition to the BSH activity and cholesterol assimilation. Moreover, all strains also possessed the properties of gut microbiota, indicating that these cholesterol-lowering probiotic LAB candidates are the probiotics potential for exerting the cholesterol reduction and health benefits on host.

Table 14 Summary the functional characteristics of cholesterol-lowering probiotic lactic acid bacteria

Functional characterization	Cholesterol-lowering probiotic LAB		
	TF-7	TF-18	TA-1
BSH activity	Strong	Strong	Moderate
Cholesterol assimilation	Strong	Strong	Moderate
Hemolytic activity	No	No	No
Antibiotic susceptibility	Good	Good	Good
GABA production	Yes	No	No
Growth characteristics	Moderate	Rapid	Slow
Antimicrobial activity	Broad spectrum	Narrow spectrum	Narrow spectrum
Adherence property	Strong	Strong	Moderate
Acid and bile tolerance	Moderate	Strong	Weak

TF-7; *Lactobacillus reuteri*

TF-18; *Enterococcus faecium*

TA-1; *Bifidobacterium animalis* subsp. *lactis*

2 Determination of cholesterol-lowering effect of candidate probiotic lactic acid bacteria on high cholesterol diet-induced rat model

2.1 Body weight of rats

During experimental procedure of rats that administered the diet with supplement, body weight of rats was determined to study the trend of lipid metabolism because body weight is a kind of parameter to indicate the metabolic abnormality. The result of body weight was presented in Table 15, body weight of 7 experimental groups increased in a time dependent manner, which their trends were similar. However, high cholesterol diet-induced rats (HF) exhibited the highest body weight.

Table 15 Body weight of rats during experimental procedure

Groups	Body weight of rats (g)				
	Week 0	Week 2	Week 4	Week 6	Week 8
NC	131.80± 10.76	212.40± 7.54	293.60± 5.94	380.60± 5.32	437.25± 5.17
HF	136.40± 5.18	243.80± 10.89	369.20± 9.07	479.20± 10.13	544.62± 20.76
NCP	133.60± 4.67	215.60± 6.99	291.60± 13.41	378.20± 4.66	449.12± 18.03
HFP1	124.80± 16.81	223.80± 6.46	305.40± 4.83	418.80± 25.37	485.98± 13.01
HFP2	136.80± 4.61	213.60± 9.42	301.60± 11.28	409.40± 6.84	491.21± 20.51
HFP3	132.80± 14.34	213.40± 11.97	304.80± 6.38	419.40± 9.34	497.67± 12.76
HFP123	136.80± 5.93	211.20± 8.76	309.40± 7.31	422.40± 4.72	470.79± 16.13

Body weight of rats was presented as mean±SD. (n=5)

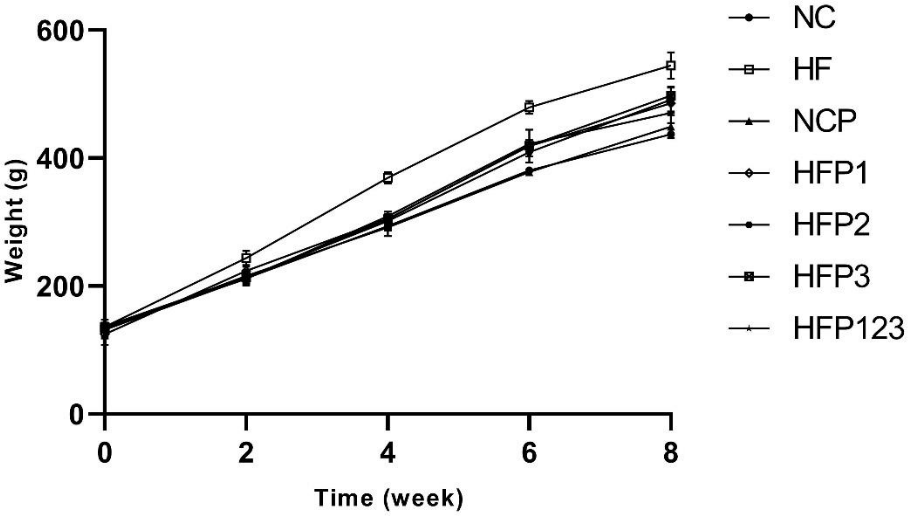


Figure 19 Body weight of rats during experimental procedure

Body weight of rats in each group was presented as mean±SD. (n=5)

2.2 Lipid profile of rats

To study the pathological process of lipid metabolism of rats after experimental procedure, 7 experimental groups were determined total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels. The lipid profiles are the clinical parameters for diagnosis of lipid metabolism. The result in Table 16 showed that TC levels of high cholesterol diet-induced rats administered with cholesterol-lowering probiotic LAB, including TF-7 (HFP1), TF-18 (HFP2), TA-1 (HFP3), and mixed three isolates (HFP123) significantly decreased ($^{\#\#\#\#}$ p < 0.0001) when compared with the high cholesterol diet-induced rats (HF), but TC levels of these experimental groups were higher than the normal diet rats (NC and NCP) with statistical significance (**** p < 0.0001). While TG levels of TF-7 (HFP1), TF-18 (HFP2), TA-1 (HFP3), and mixed three isolates (HFP123) significantly decreased ($^{\#\#\#\#}$ p < 0.0001) when compared with high cholesterol diet-induced rats (HF), but TG levels of these experimental groups were higher than the normal diet rats (NC and NCP) with statistical significance (**** p < 0.0001), which was consistent with the result of TC levels. However, HDL-C levels of TF-7 (HFP1), TF-18 (HFP2), and mixed three isolates (HFP123) was not different with statistical significance when compared with high cholesterol diet-induced rats (HF) and normal diet rats (NC and NCP). Finally, LDL-C levels of TF-7 (HFP1), TF-18 (HFP2), TA-1 (HFP3), and mixed three isolates (HFP123) significantly reduced ($^{\#\#\#}p < 0.001$) when compared with the high cholesterol diet-induced rats (HF). Whereas, when compared with normal diet rats (NC and NCP), only LDL-C levels of TF-7 (HFP1), TF-18 (HFP2), and TA-1 (HFP3) significantly elevated (Figure 20). Thus, three cholesterol-lowering probiotic LAB proved that their effect could reduce TC, TG, and LDL-C levels in the high cholesterol diet-induced rats. Additionally, they tended to synergize the effect for reducing hyperlipidemia. Therefore, the potential of these isolates was able to develop as cholesterol-lowering probiotic products.

Table 16 Lipid profile of rats after experimental procedure

Groups	Lipid profile of rats (mg/dL)			
	TC	TG	HDL-C	LDL-C
NC	85.40±8.56	89.20±6.22	40.80±6.06	26.76±8.25
HF	136.60±9.53	188.20±11.21	38.20±6.02	64.76±7.84
NCP	87.20±9.88	99.20±4.49	37.60±6.62	26.62±11.70
HFP1	113.40±6.88	126.20±6.22	38.20±5.45	45.50±11.65
HFP2	113.60±10.31	127.20±5.26	39.80±5.89	44.82±6.85
HFP3	117.80±6.38	129.40±8.96	39.20±8.11	45.78±6.90
HFP123	101.60±3.05	125.60±5.55	39.40±3.36	37.08±3.56

Lipid profile of rats was presented as mean±SD. (n=5)

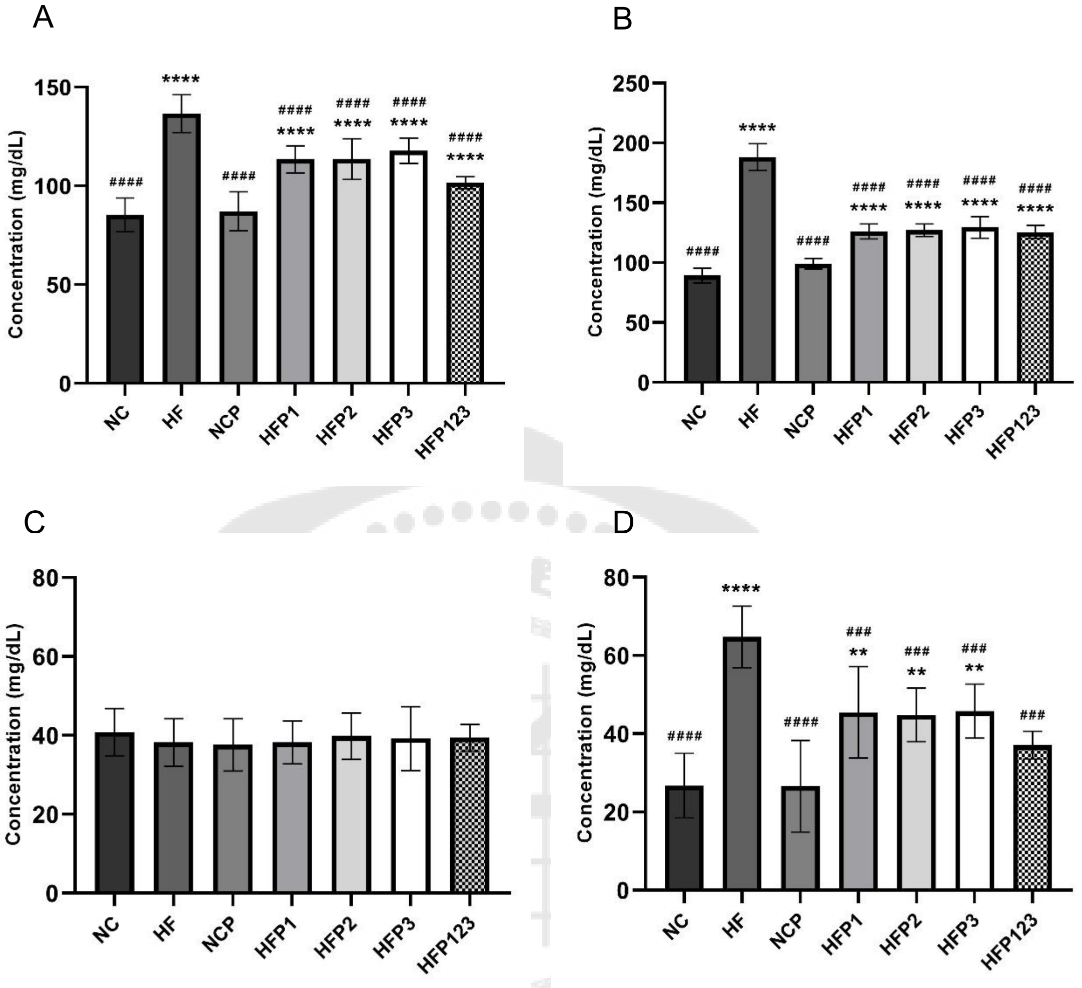


Figure 20 Lipid profile of rats after experimental procedure

(A) TC, (B) TG, (C) HDL-C, and (D) LDL-C, statistical significance of each group was compared with NC group (** $p < 0.01$, **** $p < 0.0001$) and HF group ($^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$). (n=5, mean±SD)

2.3 Gut microbiota of rats

Bacterial community in the gastrointestinal tract of rats between pre and post experimental procedure was determined to prove the modulation of biosis in the high cholesterol diet-induced rat after probiotic administration. Table 17 presented the result of gut microbiota, including lactobacilli, bifidobacteria, and *E. coli*. At the pre experimental procedure, lactobacilli, bifidobacteria, and *E. coli* of 7 experimental groups were relatively similar amount. On the other hand, gut microbiota of rat groups after experimental procedure were significantly different (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $^{\#\#\#}$ $p < 0.001$, $^{\#\#\#\#}$ $p < 0.0001$). Lactobacilli of the rats administered with cholesterol-lowering probiotic LAB, including TF-7 (HFP1) and mixed three isolates (NCP and HFP123) were higher than the rats without administering TF-7 isolate. While the amount of bifidobacteria was similar to the lactobacilli, which the rats administered with TA-1 isolate (NCP, HFP3, and HFP123) were higher amount than the rats without administering TA-1 isolate. Furthermore, the amount of *E. coli* in the rats administered with cholesterol-lowering probiotic LAB, including NCP, TF-7 (HFP1), TA-1 (HFP3), and mixed three isolates (HFP123) significantly reduced when compared with the rats without administering cholesterol-lowering probiotic LAB (NC and HF) (Figure 21). These results revealed that the rats administered with cholesterol-lowering probiotic LAB could improve the gut microbiota by increasing the amount of probiotics reducing the amount of *E. coli*, which is the enteropathogenic bacteria in the gastrointestinal tract.

Table 17 Gut microbiota of rats between pre and post experimental procedure

Groups	Gut microbiota (Log_{10} CFU/g)					
	Lactobacilli		Bifidobacteria		*E. coli*	
	Pre	Post	Pre	Post	Pre	Post
NC	8.21±0.28	8.51±0.25	6.38±0.39	8.27±0.27	6.82±0.43	7.67±0.70
HF	8.19±0.26	8.33±0.51	6.61±0.37	8.06±0.50	7.12±0.52	7.97±0.52
NCP	8.29±0.20	9.54±0.25	6.41±0.51	9.26±0.45	7.10±0.66	5.87±0.61
HFP1	8.30±0.13	9.65±0.25	5.41±0.31	7.76±0.18	7.16±0.76	6.03±0.65
HFP2	8.24±0.34	8.59±0.28	6.24±0.27	7.88±0.32	7.18±0.50	6.81±0.48
HFP3	8.31±0.08	8.51±0.31	6.39±0.29	9.63±0.27	6.86±0.72	6.29±0.72
HFP123	8.28±0.21	9.44±0.34	6.07±0.31	9.22±0.65	6.95±0.53	6.16±0.54

Gut microbiota of rats was presented as mean±SD. (n=5)

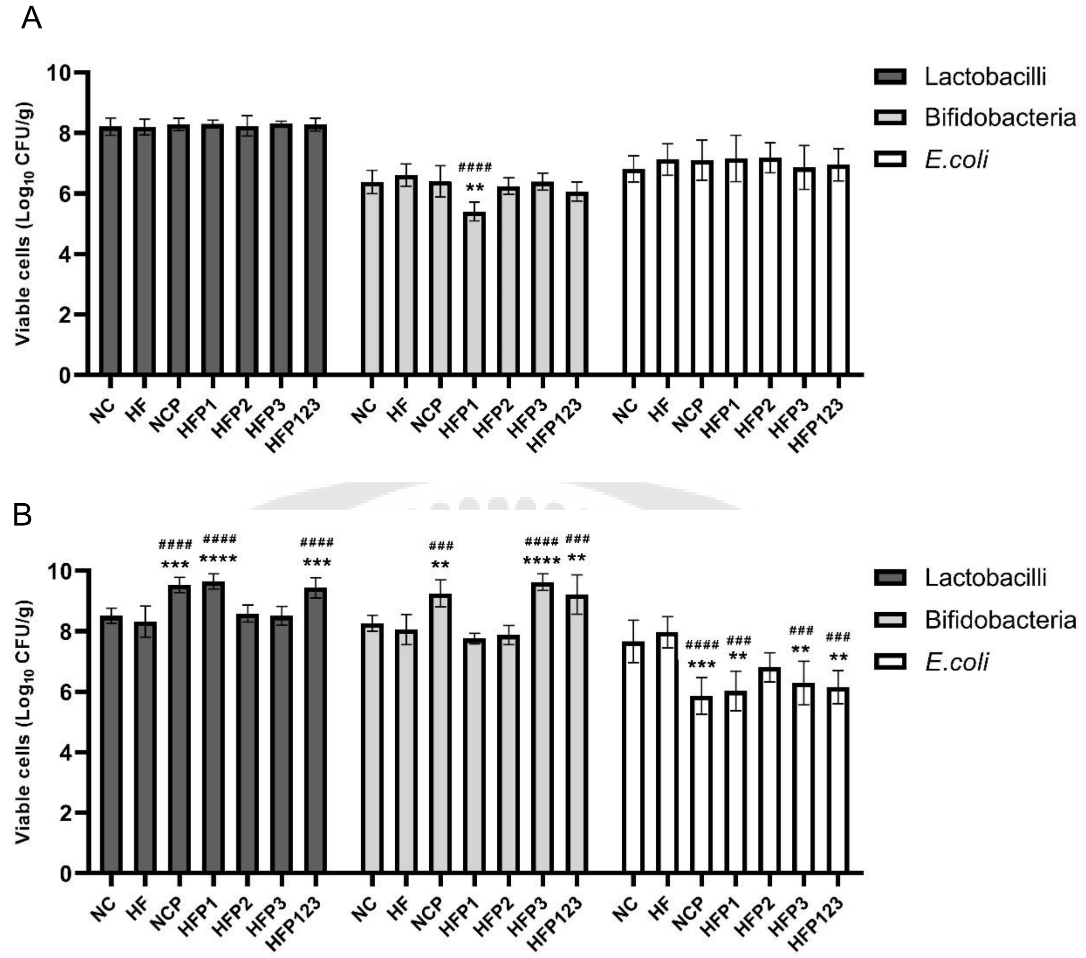


Figure 21 Gut microbiota of rats between pre and post experimental procedure

Gut microbiota of (A) pre administration and (B) post administration, statistical significance of each group was compared with NC group (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) and HF group ($^{###}$ $p < 0.001$, $^{####}$ $p < 0.0001$). (n=5, mean±SD)

3 Application of cholesterol-lowering probiotic lactic acid bacteria via microencapsulation

Cholesterol-lowering probiotic LAB after proof their potential in rat model, they were developed as the cholesterol-lowering probiotic products by spray drying microencapsulation.

3.1 Crystallinity of starch

Nano-crystalline starch (NCS) modified from cassava starch is an important wall material using in the microencapsulation. In nature, starch extracted from the cassava roots highly contains the amylose and amylopectin, whereas the amounts of lipid are much lower than other tuber starches. Consequently, these chemical compositions facilitate to modify the molecular structure for moderating of biophysical properties. Therefore, cassava starch was used as raw material for preparation by hydrolysis of starch structure. After preparation of NCS, the crystallinity of native cassava starch and cassava starch hydrolysate was analyzed using X-ray diffraction wavelength at 1.5406 angstrom and velocity of 2-Theta value was 0.02 ˚2-Theta/second. The spectrum of intensity after subtracting the background signal showed as X-ray diffractogram. Nevertheless, the spectrum still had the amorphous intensity combined with the nano-crystalline intensity, we therefore used the Gaussian method by OriginPro software version 9.1 to extract the nano-crystalline intensity. The result in Figure 22A, B, and C showed that the area under the lower graph represented the amount of nano-crystalline, but the area under the above graph represented the amount of amorphous and nano-crystalline in the starch granules, nano-crystalline content in the native cassava starch and cassava starch hydrolysate were 33.91±1.66% and 65.61±4.22%, respectively. Therefore, nano-crystalline content of cassava starch hydrolysate significantly increased after hydrolyzation of structure (**** $p < 0.0001$) when compared with the native cassava starch. Thus, cassava starch hydrolysate was suitable for use as NCS in the microencapsulation process combined with whey protein isolate (WPI).

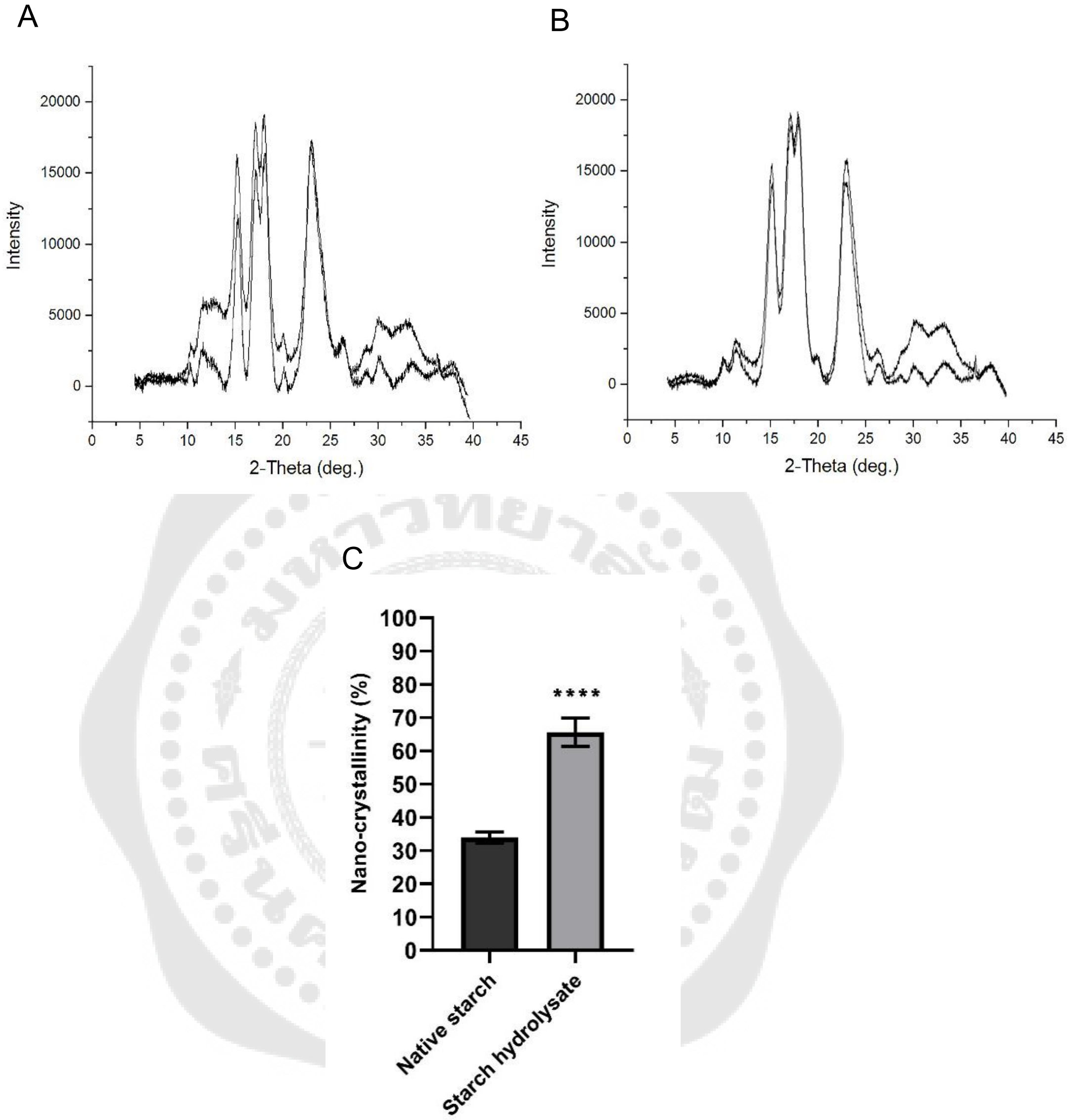


Figure 22 Crystalline analysis using X-ray diffraction

Crystallinity from (A) native cassava starch and (B) cassava starch hydrolysate, (C) the percentage of the crystallinity, statistical significance of cassava starch hydrolysate was compared with native cassava starch (**** $p < 0.0001$). (n=3, mean±SD)

3.2 Microencapsulation of cholesterol-lowering probiotic lactic acid bacteria

Microencapsulation of cholesterol-lowering probiotic LAB was performed using spray drying technique. WPI and NCS were formulate at ratio of 4:1 (WPI-NCS formula) and WPI alone (WPI formula) to encapsulate cholesterol-lowering probiotic LAB. After microencapsulation, microcapsules were studied morphology under the scanning electron microscope as shown in Figure 23. Morphology of WPI-NCS microcapsules of all three isolates was more spherical than WPI microcapsules with particle size range 4-20 um. The shrinkage of microcapsules caused by evaporation of water during spray drying. Therefore, WPI-NCS microcapsules could sustain their spherical morphology, as the wall of microcapsules were sturdy. These results indicated that particle size and shape of microcapsules were appropriate for loading bacterial cells to a few amounts per a microcapsule. Moreover, the wall structures of WPI-NCS microcapsules were rather stable during the spray drying process, demonstrating the WPI and NCS strikingly interact together as completely wall materials at an optimal ratio of 4:1. Subsequently, microencapsulation yield of encapsulated cholesterol-lowering probiotic LAB was measured as survivability of probiotic cells (Table 18). The WPI-NCS microcapsules of all three isolates were higher yield than WPI microcapsules, while microencapsulation yield of two formula was not different (Figure 24). From the trend of microencapsulation yield summarized that development of cholesterol-lowering probiotic products using spray drying microencapsulation with WPI-NCS formula were relatively high yield. Moreover, it proved that WPI and NCS incorporated as complete wall structure that relatively tolerated heat and pressure during the spray drying process in addition to as non-toxic biopolymers without adverse effect on the viability of cholesterol-lowering probiotic LAB. Further, their protective efficiency was evaluated under the conditions of simulated gastrointestinal system and long term storage.

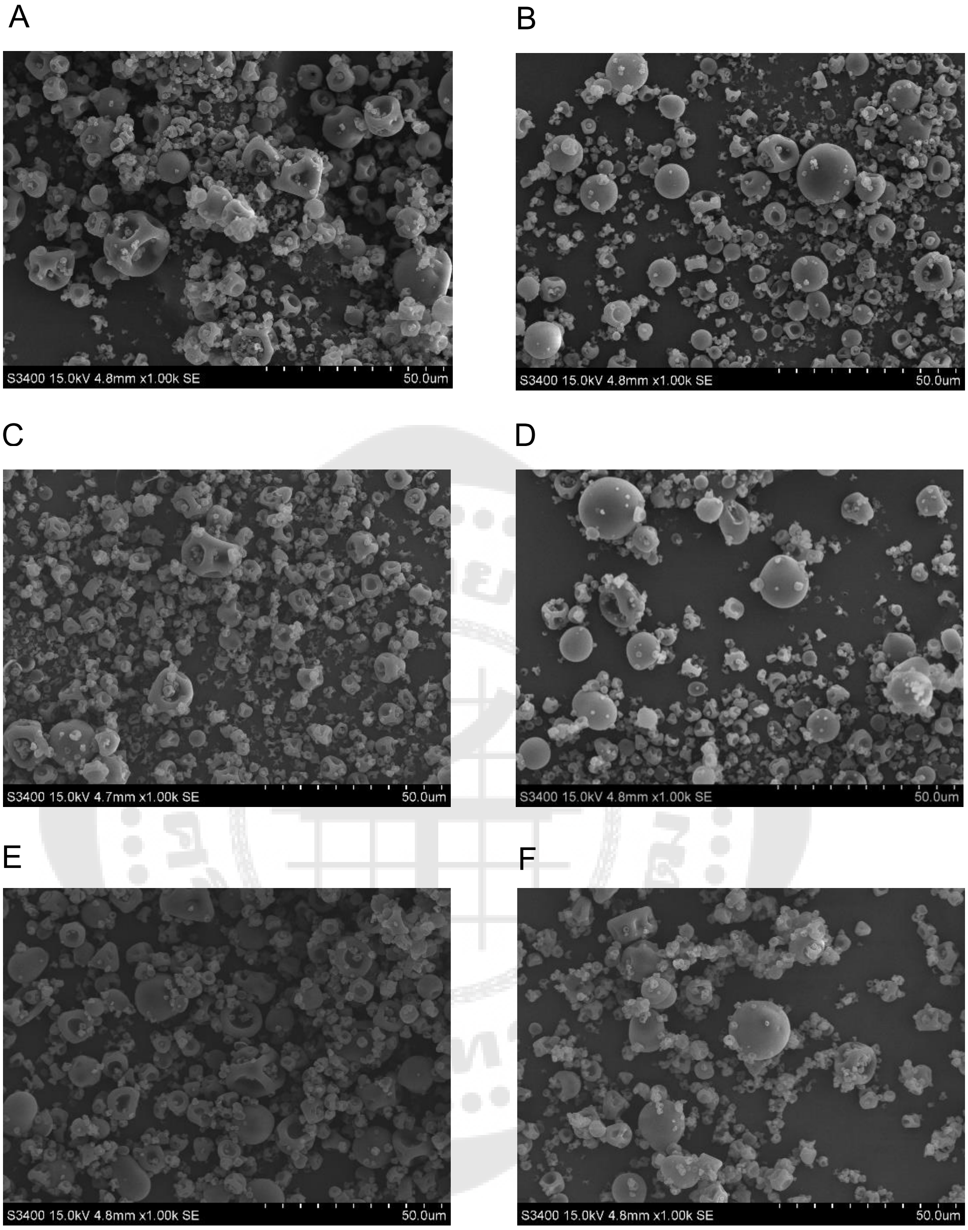


Figure 23 Morphology of microcapsules with different formula using scanning electron microscope

Morphology of microcapsule formula, (A) WPI of TF-7 isolate, (B) WPI-NCS of TF-7 isolate, (C) WPI of TF-18 isolate, (D) WPI-NCS of TF-18 isolate, (E) WPI of TA-1 isolate, and (F) WPI-NCS of TA-1 isolate. Scanning electron microscope was set at 15.0 kV high-vacuum and 1.0 k magnification.

Table 18 Microencapsulation yield of encapsulated cholesterol-lowering probiotic LAB

Microcapsule formula	Microencapsulation yield (%)		
	TF-7	TF-18	TA-1
WPI	93.28±2.69	86.15±10.33	70.07±14.57
WPI-NCS	95.83±1.53	86.64±5.80	75.26±11.77

Yield of microencapsulation was presented as mean±SD. (n=3)

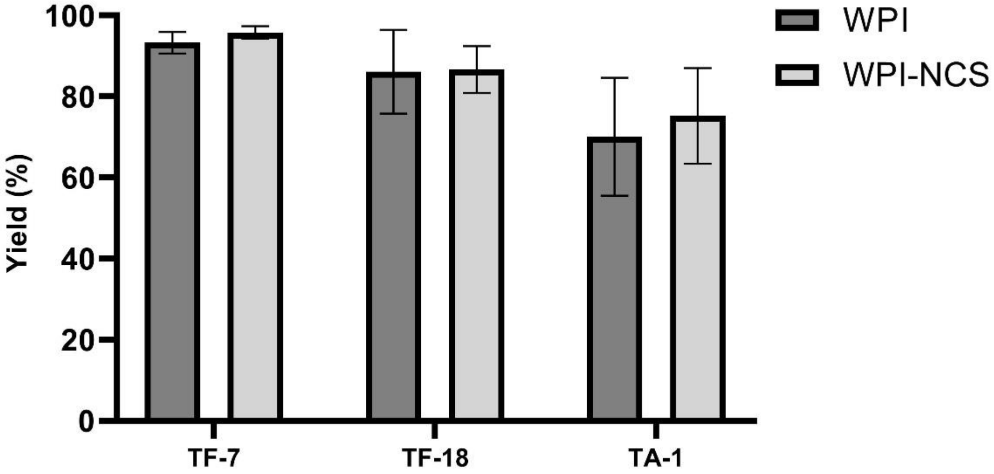


Figure 24 Microencapsulation yield of encapsulated cholesterol-lowering probiotic LAB with different formula

Statistical significance of microencapsulation yield was compared between two formula. (n=3, mean±SD)

3.3 Survivability of free and encapsulated cholesterol-lowering probiotic lactic acid bacteria in the simulated gastrointestinal conditions

Microcapsules of cholesterol-lowering probiotic LAB were determined the survivability under the simulated gastrointestinal conditions to prove the ability of microcapsules for protection on the gastrointestinal stress conditions. Stomach and intestine are negatively effects to probiotic cells, as these conditions constitute the diverse enzyme, acid, and bile salt. In this study, simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) were used as model of gastrointestinal conditions. The result from Table 19, 20, and 21 showed that survivability of free and encapsulated cholesterol-lowering probiotic LAB decreased in a time dependent manner. However, when compared between two formula of encapsulated cholesterol-lowering probiotic LAB and free cells, two formula of microcapsules were higher survival than free cells of all three isolates with statistically significance ($p < 0.05$). Moreover, WPI-NCS microcapsules were higher survival than WPI microcapsules with statistically significance ($p < 0.05$) (Figure 25). Therefore, WPI-NCS microcapsules were the most ability to protect cholesterol-lowering probiotic LAB on the gastrointestinal conditions, as encapsulating material of WPI-NCS microcapsules was suitable for resistant against the gastrointestinal conditions. While WPI microcapsules could protect cholesterol-lowering probiotic LAB to a certain extent.

Table 19 Survivability of free and encapsulated TF-7 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (Log_{10} CFU/g)		
	Free cells	WPI	WPI-NCS
0	8.74±0.17	8.73±0.06	8.71±0.10
1	5.46±0.11	6.99±0.09	7.69±0.09
2	4.74±0.08	6.01±0.14	6.98±0.04
3	3.92±0.17	5.55±0.11	6.40±0.05
4	3.55±0.09	4.96±0.09	5.86±0.11
5	3.08±0.21	4.56±0.12	5.36±0.07
6	2.64±0.11	4.03±0.18	4.88±0.14

The amount of viable cells was presented as mean±SD. (n=3)

Table 20 Survivability of free and encapsulated TF-18 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (Log_{10} CFU/g)		
	Free cells	WPI	WPI-NCS
0	8.75±0.11	8.74±0.14	8.77±0.26
1	6.58±0.13	7.03±0.09	8.05±0.09
2	5.58±0.10	6.60±0.13	7.34±0.07
3	4.89±0.06	5.70±0.16	6.78±0.08
4	4.15±0.13	5.19±0.14	6.18±0.15
5	3.65±0.07	4.82±0.05	5.48±0.15
6	3.20±0.13	4.48±0.15	5.10±0.12

The amount of viable cells was presented as mean±SD. (n=3)

Table 21 Survivability of free and encapsulated TA-1 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (Log_{10} CFU/g)		
	Free cells	WPI	WPI-NCS
0	8.78±0.08	8.69±0.08	8.80±0.19
1	4.90±0.15	6.41±0.14	7.52±0.19
2	4.16±0.09	5.39±0.27	6.72±0.08
3	3.24±0.20	4.84±0.07	5.78±0.10
4	2.59±0.20	4.71±0.13	5.25±0.08
5	2.09±0.19	4.04±0.07	4.88±0.08
6	1.65±0.10	3.65±0.11	4.37±0.09

The amount of viable cells was presented as mean±SD. (n=3)

Furthermore, the survivability of free and encapsulated cholesterol-lowering probiotic LAB on the simulated gastrointestinal conditions can analyze and express as the percentage of their survival rates. This result facilitates to observe and compare the survivability easily. As presented in Table 22, 23, and 24, the rates of survivability of three cholesterol-lowering probiotic LAB confirmed that WPI-NCS microcapsules were more effective than WPI microcapsules and free cells to protect cholesterol-lowering probiotic LAB cells under the simulated gastrointestinal conditions with statistically significance ($p < 0.05$).

Table 22 Rate of survivability of free and encapsulated TF-7 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (%)		
	Free cells	WPI	WPI-NCS
0	100±0.00	100±0.00	100±0.00
1	62.56±2.18	80.00±0.99	88.30±1.77
2	54.29±0.23	68.85±1.07	80.15±1.16
3	44.82±1.54	63.51±0.87	73.45±1.34
4	40.59±0.84	56.75±0.60	67.32±1.22
5	35.26±1.49	52.21±0.98	61.51±1.25
6	30.22±1.44	46.19±2.09	56.03±1.68

The amount of viable cells was presented as mean±SD. (n=3)

Table 23 Rate of survivability of free and encapsulated TF-18 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (%)		
	Free cells	WPI	WPI-NCS
0	100±0.00	100±0.00	100±0.00
1	75.18±0.72	80.43±0.87	91.83±2.18
2	63.82±1.75	75.52±2.00	83.72±2.89
3	55.84±0.40	65.23±0.71	77.32±2.50
4	47.47±1.68	59.44±0.97	70.57±3.05
5	41.74±0.25	55.14±0.76	62.50±1.60
6	36.61±1.36	51.27±0.81	58.25±2.69

The amount of viable cells was presented as mean±SD. (n=3)

Table 24 Rate of survivability of free and encapsulated TA-1 isolate after sequential incubation in the simulated gastrointestinal conditions

Time (h)	Survivability in the simulated gastrointestinal conditions (%)		
	Free cells	WPI	WPI-NCS
0	100.00±0.00	100.00±0.00	100.00±0.00
1	55.80±1.36	73.74±1.76	85.48±3.50
2	47.37±0.76	62.00±3.12	76.39±1.62
3	36.91±2.35	55.70±0.44	65.64±1.69
4	29.45±1.89	54.20±1.25	59.61±0.87
5	23.78±2.15	46.50±1.05	55.42±1.74
6	18.83±1.05	41.97±1.31	49.69±1.26

The amount of viable cells was presented as mean±SD. (n=3)

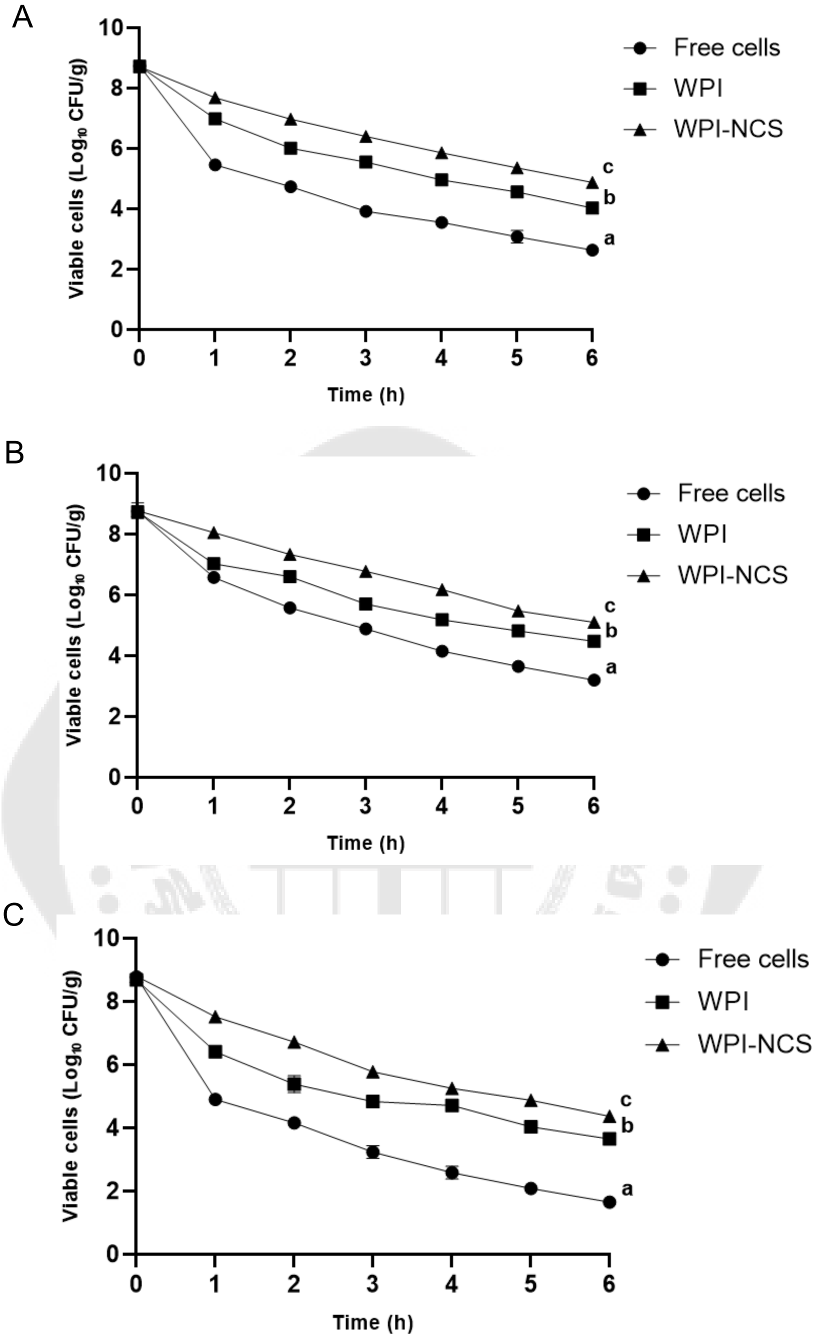


Figure 25 Survivability of free and encapsulated cholesterol-lowering probiotic LAB after sequential incubation in the simulated gastrointestinal conditions

Survivability of (A) TF-7 isolate, (B) TF-18 isolate, and (C) TA-1 isolate, statistical significance of each microcapsule formula was compared with free and other microcapsule formula (different letters indicate $p < 0.05$). (n=3, mean±SD)

3.4 Stability of free and encapsulated cholesterol-lowering probiotic lactic acid bacteria during long term storage

To maintain the biological activity of functional foods while transportation, they should be kept at 4 °C. When released products into the market, they are often on sale at ambient or refrigerated storage. Therefore, microcapsules of cholesterol-lowering probiotic LAB were stored at 4 and 25 ˚C for 12 weeks to imitate the storage conditions of commercial products for determination the survivability during long term storage. From the result in Figure 26A, 27A, and 28A presented the stability of free and encapsulated cholesterol-lowering probiotic LAB during long term storage at 4 ˚C, the stability of free and encapsulated cholesterol-lowering probiotic LAB of all three isolates decreased in a time dependent manner. However, when compared between two formula of encapsulated cholesterol-lowering probiotic LAB and free cells, two formula of cholesterol-lowering probiotic LAB were higher stable than free cells with statistically significance ($p < 0.05$). Moreover, WPI-NCS microcapsules were higher stable than WPI microcapsules with statistically significance ($p < 0.05$). While the result in Figure 26B, 27B, and 28B presented the stability of encapsulated cholesterol-lowering probiotic LAB at 25 ˚C, their result were similar with the result of stability during long term storage at 4 ˚C. Briefly, WPI-NCS microcapsules were higher stable than WPI microcapsules and free cells of all three isolates, respectively, with statistically significance ($p < 0.05$). Thus, in this study concluded that WPI-NCS microcapsules were the most stability which preserved cholesterol-lowering probiotic LAB during long term storage, as encapsulating material of WPI-NCS microcapsules was suitable for protection against the stress environments, while WPI microcapsules were stable to some extent. Furthermore, the storage temperature at 4 ˚C was more suitable than 25 ˚C to store the microcapsules of cholesterol-lowering probiotic LAB.

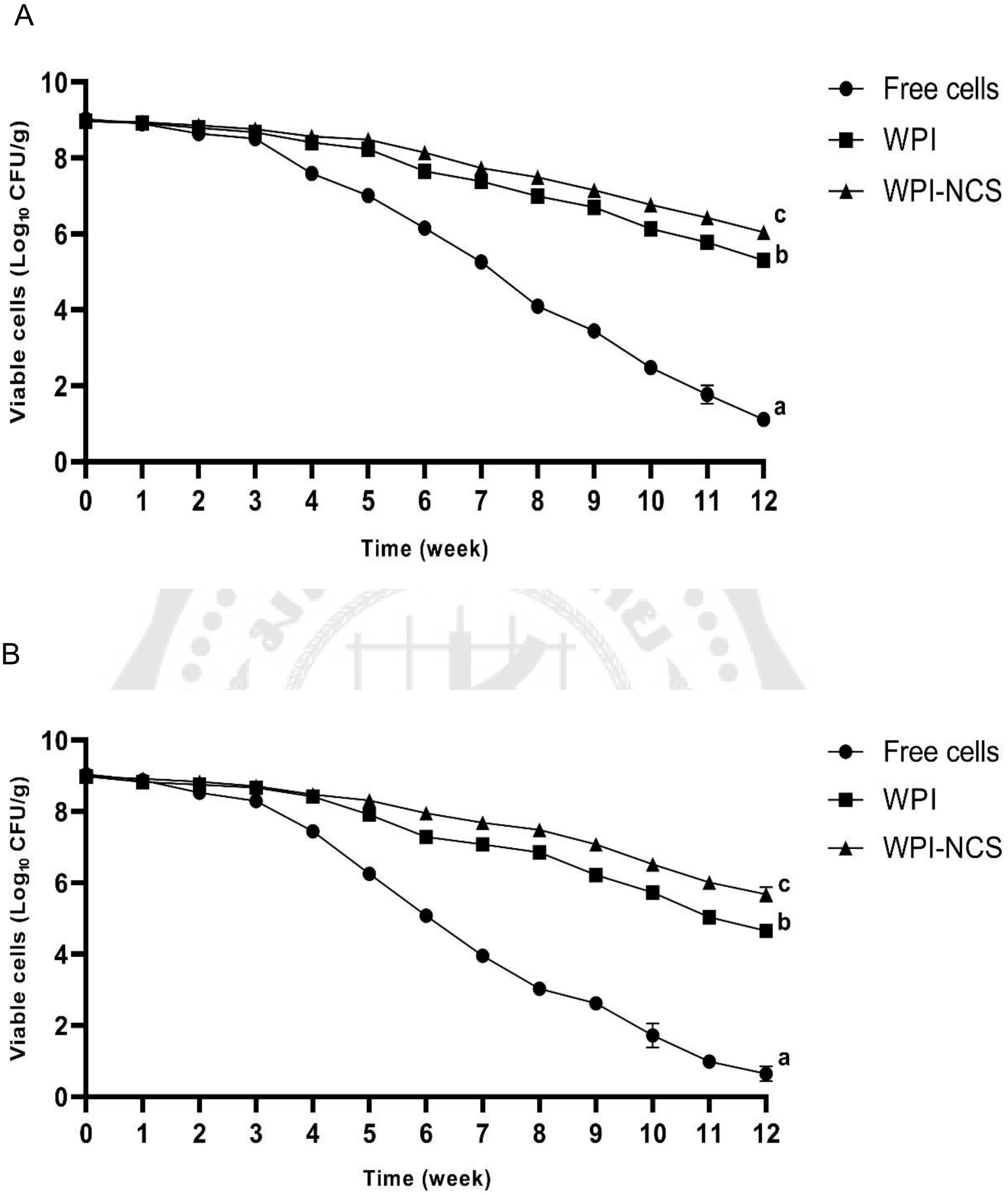


Figure 26 Stability of free and encapsulated TF-7 isolate during long term storage

Stability at (A) 4 ˚C and (B) 25 ˚C, statistical significance of each microcapsule formula was compared with free and other microcapsule formula (different letters indicate $p < 0.05$). (n=3, mean±SD)

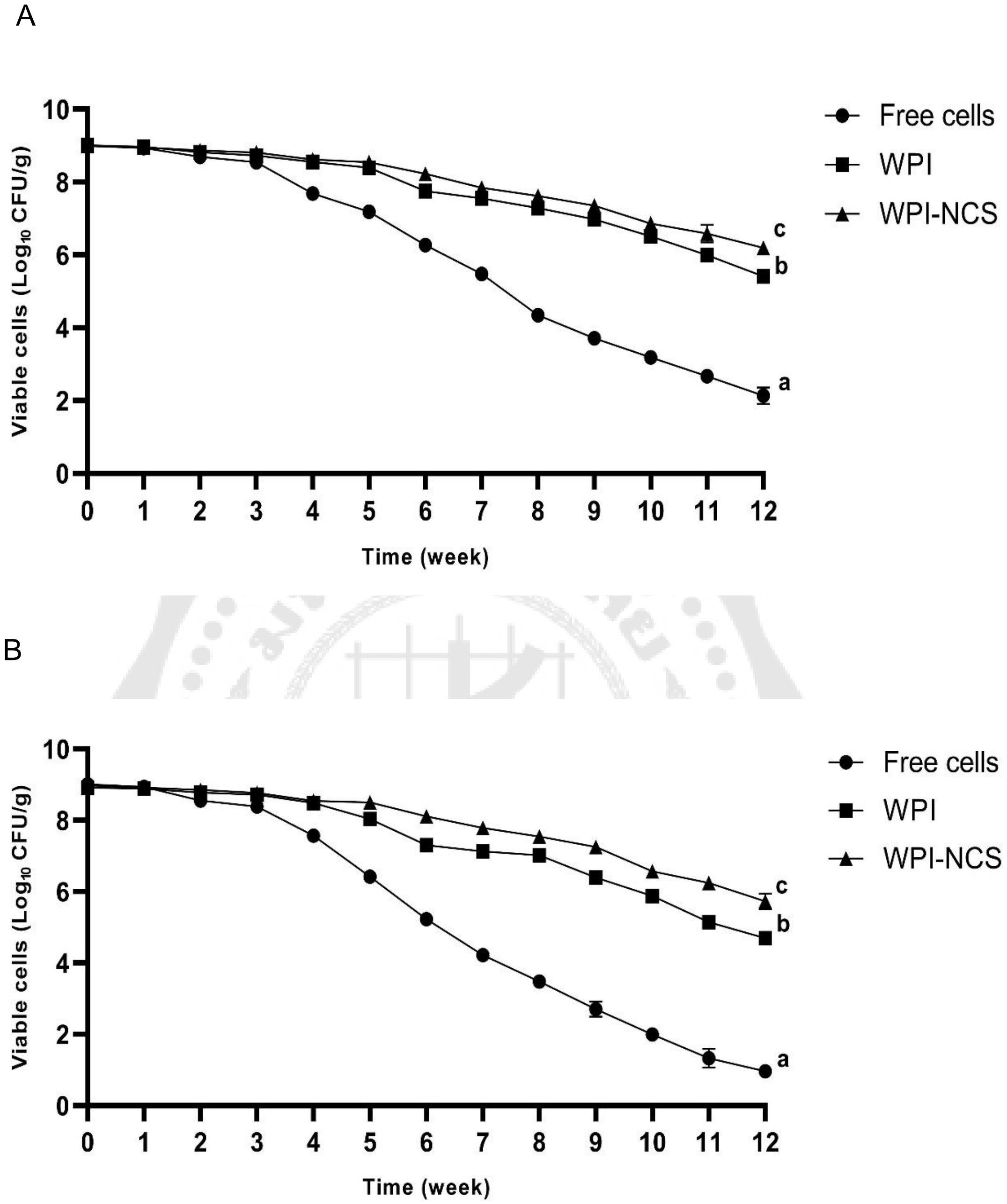


Figure 27 Stability of free and encapsulated TF-18 isolate during long term storage

Stability at (A) 4 ˚C and (B) 25 ˚C, statistical significance of each microcapsule formula was compared with free and other microcapsule formula (different letters indicate $p < 0.05$). (n=3, mean±SD)

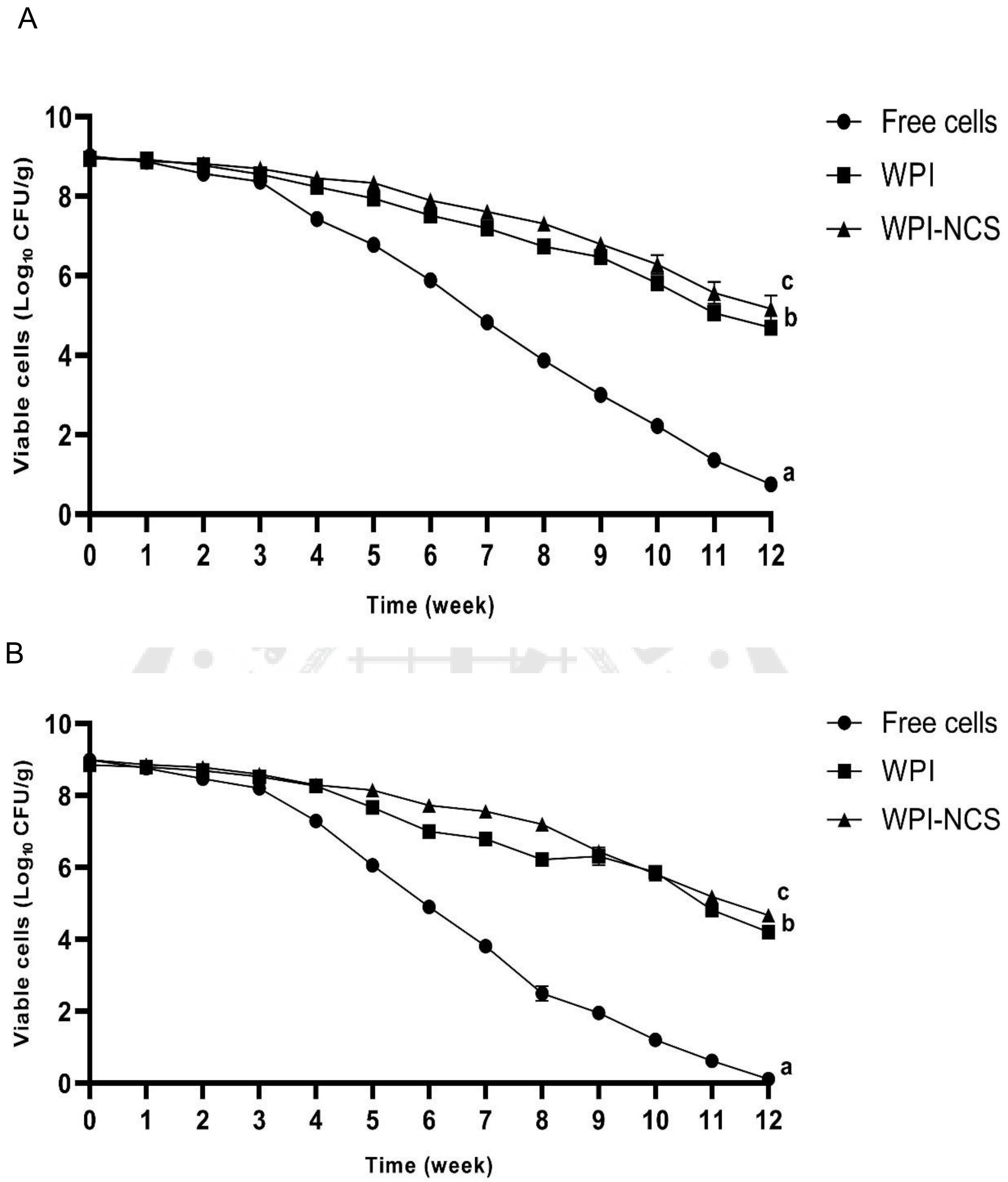


Figure 28 Stability of free and encapsulated TA-1 isolate during long term storage

Stability at (A) 4 ˚C and (B) 25 ˚C, statistical significance of each microcapsule formula was compared with free and other microcapsule formula (different letters indicate $p < 0.05$). (n=3, mean±SD)

CHAPTER 5

DISCUSSION

Probiotic lactic acid bacteria (LAB) are live microorganisms that extensively exhibit the crucial activity for health promoting effect in the human and animal. The important organ system for probiotic establishment is the gastrointestinal system because it contains the essential nutrients for growth promotion of microorganisms, especially the intestine.(120) For this reason, the intestine is found the diversity of bacterial communities, which is the gut microbiota. However, there are two major group of bacteria such as probiotics, normal flora, and pathogens. Probiotics and pathogens play a vital role in an indicator of health status.(121) Thus, the regulation of homeostasis between probiotics and pathogens is the important aspect. The replenishment of probiotic LAB with oral administration is the easiest modulation of gut microbiota to promote health status. Therefore, the functional characteristics, safety, and the acid and bile tolerance in the gastrointestinal conditions are the crucial criteria of probiotics prior to application as the probiotic products, according to the specification of GRAS.(122) Additionally, probiotics from the gut microbiota plays a fundamental role in facilitating nutrient metabolism and improvement of gastrointestinal function. The cholesterol-lowering activity is a specific property of probiotics, which contributes to the modulation of cholesterol metabolism. Therefore, the cholesterol-lowering effect is able to use for prevention and reduction the risk of metabolic disorder caused by cholesterol and dyslipidemia. There are several previous studies elucidated the cholesterol-lowering mechanisms of probiotics that bile salt hydrolase (BSH) enzyme is the indirect mechanism to reduce cholesterol absorption because it is able to hydrolyze conjugated bile salt into deconjugated bile salt, resulting in deficient absorption of lipid.(123) Additionally, cholesterol in the body is consumed for synthesis of new bile salt through de novo pathway. The direct mechanism is an assimilation of cholesterol, it can consume cholesterol and other lipids in the lumen of intestine to produce their molecules and cell structure.(124) Moreover, the diversity of probiotic LAB can synergize the cholesterol-lowering effect when administered simultaneously. Thus, it is importance to select the different genera of cholesterol-lowering probiotic LAB to apply as probiotic products. However, there are several factors in the industrial processes and storage of probiotic products that negatively affect on the survivability and stability of probiotic products.(125) Presently, the development of food products have used various biotechnologies to apply in the food production processes. Microencapsulation is a biotechnology to preserve the unstable substances with encapsulating materials.(126) Thus, probiotics LAB can apply as probiotic products using microencapsulation to enhance the survivability and stability of probiotic LAB.(127) Therefore, the purposes of this study were to isolate three genera of probiotic LAB including *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. with ability to reduce cholesterol and express the functional characteristics of probiotics. These genera of cholesterol-lowering probiotic LAB were developed for probiotic products via spray drying microencapsulation with whey protein isolate (WPI) and nano-crystalline starch (NCS) to enhance the survivability under the gastrointestinal conditions after administration and stability during storage of probiotic products.(128)

In this study, probiotic LAB were isolated from 50 traditional food samples. However, there were only 74 probiotic LAB isolates with distinct morphology showed the desired characteristics. Subsequently, all 74 probiotic LAB isolates were determined the motility because the bacterial motility use cillia or flagella, which is virulence factor of pathogens. From the result showed all 74 probiotic LAB isolates was not motile, indicating that they do not have the cilia or flagella which are the virulence factor of pathogens to invade the host tissues. Thus, all probiotic LAB isolates were able to prove for the ability to reduce cholesterol by BSH production and cholesterol assimilation. Their BSH activity were investigated with taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA) because they are the major conjugated bile salt that secrete to intestinal lumen, which is hydrolyzed by BSH of probiotic LAB in the gut microbiota.(129) The result of BSH activity showed different activity in 17 probiotic LAB isolates because it can recognize both taurine and glycine group or only one to hydrolyze amide bond between steroid molecule and amino acid. However, probiotic LAB with ability to express BSH activity both TDCA and GDCA were the dominant isolates of BSH production (Figure 12). BSH from probiotics was different activities because it can recognize both taurine and glycine group or one to hydrolyze amide bond between steroid nucleus and amino acid side chain, which is strain dependent.(130) The cholesterol assimilation was investigated the direct mechanism of *in vitro* cholesterol reduction.(131) Cholesterol assimilation showed different abilities to utilize cholesterol, which was consistent with some study in which the cholesterol assimilation is depends on the individual cellular activities.(132) Obviously, TF-17 and TF-18 isolates were the strongest assimilation of cholesterol (more than 50% cholesterol assimilation), which accord their BSH activity.

Notwithstanding, all 17 probiotic LAB isolates were confirmed the species of probiotics with 16s ribosomal RNA (rRNA) gene sequencing, and all probiotic LAB isolates belonged to four genera (Table 8), including *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*. The probiotic LAB species with ability to produce BSH which listed in the Food and Drug Administration (FDA) of Thailand such as *L. reuteri*, *E. durans*, *E. faecium*, *L. gasseri*, *B. animalis* subsp. *lactis*, and *L. johnsonii*. In this study, we selected different three genera, including *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* subsp. *lactis* TA-1 for candidate of cholesterol-lowering probiotic LAB, considering with their cholesterol-lowering effect both BSH activity and cholesterol assimilation, compared with similar genera. Obviously, *Lactobacillus* was the major genus, which was in agreement with previously reported that *Lactobacillus* was the major genus of probiotics because it was the facultative anaerobe with rapid propagation. While TA-1 isolate was selected for candidate of cholesterol-lowering probiotic LAB because it was an important genus of gut microbiota in the healthy animal and human origin with exerting various health benefits.(133)

Three genera of cholesterol-lowering probiotic LAB were further evaluated safety for use, functional properties, gastrointestinal tolerance, and adherence ability. These are the essential features of probiotic LAB to develop as probiotic products.

Hemolytic activity and antibiotic susceptibility were performed to assess the safety of cholesterol-lowering probiotic LAB. Even though there is rare evidence to indicate that probiotics are the cause of disorders, but to confirm that they do not exhibit the pathological effects on host.(134) The investigation of hemolytic activity was used to detect hemolytic toxin of bacteria because this toxin is a virulence factor to destruct the plasma membrane of erythrocyte, which contributes to the hematological disorders.(135) The result of hemolytic activity represented that three isolates could not hemolyze, but they should be further investigate virulence genes for confirmation (Figure 14). Moreover, the antibiotic susceptibility is the significant test to indicate the safety of three isolates for use in the human.(136) From this result concluded that three isolates were resistant rates less than 25% (Table 9). However, penicillin was the most resistant antibiotics, while vancomycin was resisted from TF-18 and TA-1 isolates because *Enterococcus* is a common resistant genera which calls vancomycin-resistant enterococci.(137) There are some evidences reported that *Bifidobacterium* was resistant with vancomycin because it can receive the plasmid with resistant gene from other bacteria in the bacterial community.(138) From the result of hemolytic activity and antibiotic susceptibility indicates that three isolates are safety to a certain extent for development as probiotic products using in the human.

However, probiotic LAB for development as the probiotic products should express various health promoting properties in addition to cholesterol reduction. The biosynthesis of gamma-amino butyric acid (GABA) is a specific property of probiotic LAB in the gut microbiota because the nutrients in the gastrointestinal environment can serve as vital substances for production the biological molecules.(139) Thus, probiotic LAB with ability to express glutamic acid decarboxylase (GAD) can produce GABA from glutamic acid by their GAD activity.(140) From the result in Figure 15 showed that only TF-7 isolate exhibited GABA biosynthesis form glutamic acid and monosodium glutamate. Therefore, TF-7 isolate is able to assimilate glutamic acid and monosodium glutamate from nutrients to produce GABA. GABA is the neurotransmitter of enteric-nervous system that can improve the gastrointestinal function by through the modulation of autonomic nervous system.(141) There are previous studies reported that probiotic LAB producing GABA could enhance the process of gastrointestinal digestion and prevent the constipation. Additionally, GABA could persist the development of neuro-degeneration caused by antioxidant activity and regulation of neurological disorders.(142, 143)

When studied the growth characteristics of three isolates found that TF-18 isolate was faster grew than other isolates, while TA-1 isolate was the slowest growth among other isolates (Figure 16). These results supported that *B. animalis* subsp. *lactis* TA-1 was relatively low found in food samples, as their proliferation rates was slow. Additionally, the early stationary phase was interested in this study because this growth period of probiotic cells was used to apply in the microencapsulation process due to the highest cell propagation and activity.

Another important property of probiotic LAB is the ability to inhibit the growth of pathogens. It is basically desired activity of probiotics within the gut microbiota. Twelve pathogens were used to test the antimicrobial activity of cholesterol-lowering probiotic LAB, and all three isolates could inhibit the growth of pathogen at least three species, which TF-7 isolates expressed the strongest antimicrobial activity (Table 10). Therefore, three isolates can colonize with competitive exclusion against various pathogens, which play an important role in the modulation of the gut microbiota.(144) The administration of three isolates can also prevent diarrhea caused from dysbiosis.(145)

After investigation the functional properties of three cholesterol-lowering probiotic LAB, they were determined growth characteristics under the gastrointestinal conditions, it is an essential characteristics of probiotic LAB in the gut microbiota.(146) Adherence property was performed to evaluate the ability of colonization, Caco-2 cells were used for model of intestinal epithelial cells with *L. rhamnosus* GG as a positive control.(147, 148) The result of adherence property showed that three isolates were able to adhere on the Caco-2 cells with the amount of adhered cells at least 6.50 Log_{10} CFU/mL, and only the adherence ability of TA-1 isolate was lower than *L. rhamnosus* GG (Figure 17). Thus, all three isolates can adhere on the intestinal epithelial cells for their colonization as probiotic LAB in the gut microbiota to promote gastrointestinal function. However, the adherence ability of these strains was different because it depends on surface hydrophobicity and auto-aggregation of each strain.(149, 150) Acid and bile tolerance are another the significant characteristics of probiotic LAB in the gastrointestinal tract because they are administered passage through gastric and intestinal environment which negatively affect to survivability of probiotic LAB.(151) Acidity can denature intracellular protein, while bile salts are detergent molecule that can leach phospholipid in the plasma membrane. In this study, acid and bile were prepared with range of pH at 2.0 to 4.0 and range of bile concentration at 0.3 and 0.8% (w/v) which emulate the physiological conditions of gastrointestinal tract.(152) After three isolates exposed with different acid and bile conditions, bacterial survival significantly decreased in a concentration-dependent manner (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$), compared with unadjusted MRS control. The reference strain *L. rhamnosus* GG also expressed the trend of acid and bile tolerance similar to cholesterol-lowering probiotic LAB (Figure 18). However, the survivability of three cholesterol-lowering probiotic LAB under the most stress conditions at pH 2.0 and 0.8% (w/v) bile was higher than 3.40 Log_{10} CFU/mL and 7.40 Log_{10} CFU/mL, respectively. Therefore, after administration of cholesterol-lowering probiotic LAB passage through gastrointestinal tract, their survivability gradually decreased, but the amount of viable cells are sufficient to a certain extent for establishment of the gut microbiota in the human.

Three cholesterol-lowering probiotic LAB with ability to produce BSH, assimilate cholesterol, and express functional properties were confirmed the cholesterol-lowering effect and the modulation of gut microbiota in rat model. Thirty-five male wistar rats aged 8 weeks were randomly assigned into 7 experimental groups as described in the method of animal housing and feeding. They were treated with diet and supplements as summarized in Table 4. The probiotic LAB isolates 1, 2, and 3 were represented as *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* subsp. *lactis* TA-1, respectively.(153)

During experimental procedure, the body weight of rats in each group was determined to indicate pathological process of cholesterol metabolism because the body weight is a parameter of metabolic disorders caused by hypercholesterolemia. The body weight of all experimental groups increased in a time dependent manner. Obviously, the body weight of high cholesterol diet-induced rats (HF) was higher than the other groups (Figure 19). Our findings suggested that high cholesterol diet could induced the body weight gain, but the administration of cholesterol-lowering probiotic LAB could prevent the obesity.(154)

Therefore, the lipid profile of rats was analyzed after experimental procedure to confirm the hypocholesterolemic effect of probiotics, as it is an important to indicate the therapeutic effect after administration of cholesterol-lowering probiotic LAB. The result from Table 16 summarized that high cholesterol diet-induced rats administered with cholesterol-lowering probiotic LAB, including TF-7 (HFP1), TF-18 (HFP2), TA-1 (HFP3), and mixed three isolates (HFP123) could significantly reduce total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) levels with similar to normal diet rats (NC and NCP), and their lipid profiles were lower levels than the high cholesterol diet-induced rats (HF) (** $p < 0.01$, **** $p < 0.0001$, $^{\#\#\#}$ $p < 0.001$, $^{\#\#\#\#}$ $p < 0.0001$). However, high-density lipoprotein cholesterol (HDL-C) levels of all groups were similar levels because HDL-C levels obviously present the different levels when prolong experimental period. Furthermore, the lipid profile of high cholesterol diet-induced rats administered with the mixed three isolates (HFP123) tended to decrease when compared with high cholesterol diet-induced rats administered with single TF-7 (HFP1), TF-18 (HFP2), and TA-1 (HFP3), respectively (Figure 20). The mixed three isolates could reduce only TC and LDL-C because they are the cholesterol lipoprotein derivative. Therefore, they were reduced by BSH activity and lipid assimilation. These results indicated that the simultaneous administration of three isolates could synergize their cholesterol-lowering ability without adverse effects. Similarly, previous study reported that the oral administration of several probiotic strains was relatively high therapeutic effect to reduce hyperlipidemia and hepatic steatosis by BSH activity and cholesterol assimilation.(155)

Previous studies have described that probiotics could improve gut microbiota in order to exert health-promoting effect and eliminate the risk of diseases. Thus, gut microbiota of high cholesterol diet-induced rats between pre and post experimental procedure was determined to prove the association of bacterial amount in the gastrointestinal tract and metabolic disorders. In the study, lactobacilli, bifidobacteria, and *E. coli* were the representative bacteria because they are mostly found in the gut microbiota and influence to regulate the nutrient metabolism of host.(156) The result in Table 17 showed that the amount of gut microbiota at pre experimental procedure were similar because all rats were not administered with cholesterol-lowering probiotic LAB and they were grew under the same conditions before experimental procedure. At post experimental procedure displayed that the amount of lactobacilli and bifidobacteria in rats administered with cholesterol-lowering probiotic LAB were elevated with statistical significance (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $^{###}$ $p < 0.001$, $^{####}$ $p < 0.0001$) when compared with rats administered without cholesterol-lowering probiotic LAB. However, the number of *E. coli* in rats administered with cholesterol-lowering probiotic LAB was obviously reduced. It caused by the antimicrobial effect after administration the cholesterol-lowering probiotic LAB (Figure 21). Therefore, the administration of cholesterol-lowering probiotic LAB could modulate the gut microbiota to a certain extent and prevent dysbiosis. Considering the results of lipid profile and gut microbiota, these results are associated together because the rat groups with low levels of lipid profile were relatively high amount of probiotics. Therefore, the administration of three cholesterol-lowering probiotic LAB could enhance the gut microbiota, which contributed to reduction of cholesterol. They are the potential biotherapeutics to develop as the probiotic supplements for reducing the risk of metabolic disorders caused by hypercholesterolemia.(157, 158)

After investigation *in vivo* hypocholesterolemic potential of cholesterol-lowering probiotic LAB, they were further applied as the cholesterol-lowering probiotic products by spray drying microencapsulation in order to produce the dry powder microcapsules. In this study, WPI was used as encapsulating material combined with NCS because WPI is a by-product of cheese with high nutrient values, as it contains more than 90% of total protein.(159) While NCS is a modified starch from cassava that can tolerate against the digestion of gastric acid and hydrolytic enzymes.(160, 161) As the molecular structure, WPI contains various amino acids which appear the nitrogen and oxygen atom for bonding with cabonyl group of sugar molecule in the oligosaccharide by intermolecular glycation.(162) This reaction is the glycosylation process which occur under the high temperature with slight basic condition (pH 7.5-8.0). Thus, the Maillard reaction of microencapsulation is the most suitable process for encapsulating the cholesterol-lowering probiotic LAB by WPI and NCS, which the molecular structure of NCS can sturdily attach with amino acid of WPI.(163, 164)

However, NCS is relatively low amount in the native starch granules, NCS is therefore prepared to hydrolyze an amorphous structure of starch granules to increase the content of nano-crystalline structure. For preparation of NCS from cassava starch, sulfuric acid was used to hydrolyze the structure of cassava starch because it is a strong acid that can completely convert nano-crystalline structure of native cassava starch.(165) The cassava starch hydrolysate was analyzed the nano-crystalline content by X-ray diffraction (Figure 22).(166, 167) Nano-crystalline content of cassava starch hydrolysate significantly increased (**** $p < 0.0001$) when compared with native cassava starch. From this result indicated that hydrolyzation of starch granules with sulfuric acid could modify native cassava starch to enhance the crystallinity. Furthermore, the structure of cassava starch hydrolysate with high content of nano-crystals could be potentially used as an encapsulating material combined with WPI for spray drying microencapsulation because the molecular structure of nano-crystals contains the short chain amylopectin that discloses the hydrogen bond to interact with the protein biopolymers by glacosylation.

WPI and NCS were used to encapsulate the cholesterol-lowering probiotic LAB in order to produce microcapsules. These encapsulating materials were formulated at WPI-NCS (4:1) and WPI alone to compare their efficacy for enhancement the survivability and stability of encapsulated cholesterol-lowering probiotic LAB. The conditions of microencapsulation process is the Maillard reaction.(168) At this condition, pH and temperature facilitate to modify the chemical characteristics of WPI, especially beta-lactoglobulin and alpha-lactalbumin, to interact with the molecular structure of NCS. As temperature increased to 90 ˚C for 10 min, globular proteins initiate unfolding and disclose the amine group facilitating to recognize with hydroxyl and carbonyl group of reducing sugar. When adjusted pH to 7.5, the surface charges of globular protein structure are changed, which is able to form with the intermolecular glycation, resulting in polymerization between WPI and NCS as homogeneous encapsulating materials.(169) Moreover, our preliminary data showed the addition of gum arabic could maintain the colloidal condition more than xanthan gum and guar gum without the interference on the intermolecular cross links between WPI and NCS and it could protect the aggregation of microcapsules. Thus, gum arabic was used in the microencapsulation process. Meanwhile, the spray drying microencapsulation was performed under the conditions at 120 ˚C inlet temperature, 75 ˚C outlet temperature, and flow rate of 0.27 L/h, which is the optimal conditions for these encapsulating materials.(170)

After microencapsulation, WPI-NCS and WPI microcapsules of three cholesterol-lowering probiotic LAB were studied the morphology under scanning electron microscope at 1.0 k magnification. Figure 23 displayed the morphology of encapsulated cholesterol-lowering probiotic LAB between WPI-NCS and WPI formula, these morphologies presented that two microcapsule formulas were spherical shape with various particle size about 4-20 um. Comparing the morphology of microcapsules between two formula, it indicated that the morphology of WPI microcapsules shrank more than WPI-NCS microcapsules, as the shrinkage occurred from the evaporation of water in the microcapsules during spray drying process.(171) From this reason concluded that WPI-NCS microcapsules were sufficient strength to support microcapsule structure. However, the various sizes of microcapsule caused by the conditions of spray drying conditions, especially spray nozzle and air pressure, which might affect to a reduction of the protective efficacy of microcapsules. To prove the durability of microcapsules for protection the cholesterol-lowering probiotic LAB during spray drying microencapsulation process, viable cells of encapsulated cholesterol-lowering probiotic LAB were measure after microencapsulation (Table 18). WPI-NCS microcapsules exhibited viable cells of three isolates higher than WPI microcapsules (Figure 24). It proved the WPI-NCS microcapsules were durable for protection the viability of three cholesterol-lowering probiotic LAB, while WPI microcapsules were also durable to some extent. However, encapsulated TA-1 isolate was the lowest yield because *Bifidobacterium* is an obligate anaerobe with high sensitivity to oxygen and heat conditions.(172)

The survivability under the gastrointestinal conditions and stability during long term storage are the important parameters to indicate the protective efficacy of microcapsules for improvement the essential features of commercial probiotic products.(173, 174) The survivability of encapsulated cholesterol-lowering probiotic LAB was studied under the simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) because they has the most negative effect on survival rate of cholesterol-lowering probiotic LAB after oral administration.(175) The gastrointestinal juices were simulated according to physiological conditions of human gastrointestinal tract, which consist of gastric acid, bile, and several hydrolytic enzymes.(176) After incubation of encapsulated three isolates under the simulated gastrointestinal conditions, the survivability of encapsulated three isolates gradually decreased in a time dependent manner (Table 19, 20, and 21). Also, WPI-NCS microcapsules were higher survival than WPI microcapsules and free cells, respectively ($p < 0.05$) (Figure 25). From this result summarized that WPI-NCS microcapsules could significantly enhance survivability of cholesterol-lowering probiotic LAB better than WPI microcapsules. Furthermore, WPI combined with NCS is the encapsulating materials that appropriate for liberation of entrapped probiotics from microcapsules. Since the intermolecular cross-linking in the microcapsule wall may retard the digestion of enzymes.(177) WPI is partially digested with the gastric enzymes, but its buffering capacity facilitates to neutralize the gastric acid for maintaining some intact microcapsules.(178) While the modified molecular structure of NCS relatively resists the digestion of gastric enzymes leading to promotion the resistant ability.(179) For intestinal transit, the encapsulating materials may be digested by the intestinal conditions, and the entrapped probiotics were therefore released in the suitable site of the gut microbiota.(180) Therefore, the microcapsule structure of WPI-NCS formula is tolerant to the stress condition in the gastrointestinal tract and suitable for probiotic liberation.

The stability of encapsulated cholesterol-lowering probiotic LAB was investigated during long term storage at 4 ˚C and 25 ˚C for 12 weeks, which imitate the ambient and refrigerated storage conditions of commercial products. This study showed the stability of encapsulated three isolates gradually decreased in a time dependent manner (Figure 26, 27, and 28). When compared between two formula of microcapsule and free cells, WPI-NCS microcapsules were higher stable than WPI microcapsules and free cells, with statistically significance ($p < 0.05$). WPI-NCS microcapsules were therefore suitable than WPI microcapsules. Moreover, WPI-NCS microcapsules could protect encapsulated cholesterol-lowering probiotic LAB from environmental factors that affect to viability and functional properties during storage of probiotic products. Moreover, the storage condition at 4 ˚C could extend shelf life more than 25 ˚C to store the survival of cholesterol-lowering probiotic LAB. Considering the survivability under the gastrointestinal conditions and stability during long term storage of encapsulated cholesterol-lowering probiotic LAB, these results were in agreement with the previous study that reported the WPI in combination with carbohydrate biopolymers was able to enhance the survivability and stability of probiotics.(181, 182) Furthermore, encapsulated TF-18 isolate was higher survival than encapsulated TF-7 and TF-8 isolate, respectively. The different survivability and stability of encapsulated cholesterol-lowering probiotic LAB influence from individual growth characteristics of each isolate.(183) These findings suggested that the spray drying microencapsulation with WPI-NCS formula is the most robust efficiency for development the cholesterol-lowering probiotic LAB products, which enhance the survivability under the gastrointestinal conditions after administration and stability during long term storage of probiotic products effectively.

CONCLUSIONS

The present study, we found 74 probiotic lactic acid bacteria (LAB) isolates with distinct morphology showed the basically desired characteristics. Interestingly, 17 probiotic LAB isolates exhibited the cholesterol-lowering activity both bile salt hydrolase (BSH) activity and cholesterol assimilation. However, three genera of probiotic LAB were selected for the candidate of cholesterol-lowering probiotic LAB based on the most robust BSH activity and cholesterol assimilation, which listed in the list of Food and Drug Administration (FDA) of Thailand. Three isolates, including TF-7, TF-18, and TA-1 isolates were defined following strain names: *Lactobacillus reuteri*, *Enterococcus faecium*, and *Bifidobacterium animalis* subsp. *lactis*, respectively. Moreover, they strikingly expressed the functional properties of probiotics with different characteristics. When evaluated the growth characteristics under the gastrointestinal conditions, all isolates partially tolerate under the most stress acid and bile conditions and also adhere to the intestinal epithelial cells for colonization in the gastrointestinal tract for exerting health benefits.

In vivo study, three cholesterol-lowering probiotic LAB extensively exhibited hypocholesterolemic effect and promote the amount of probiotic LAB in the gut microbiota. Therefore, administration of three isolates could prevent the metabolic disorders caused by hypercholesterolemia.

Three cholesterol-lowering probiotic LAB could be potentially used to develop as probiotic products by spray drying microencapsulation with whey protein isolate (WPI) and nano-crystalline starch (NCS). Our findings indicated that the spray drying microencapsulation with WPI and NCS at formula of 4:1 was the most potential to enhance the survivability under the gastrointestinal conditions and stability during long term storage. Therefore, three cholesterol-lowering probiotic products are useful as the biotherapeutic strategy for eliminating the risk of metabolic disorders caused by cholesterol. Further, the clinical trial is necessary to prove hypocholesterolemic effect of three cholesterol-lowering probiotic LAB.

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