

คุณลักษณะและการพัฒนาสูตรผลิตภัณฑ์โพรไบโอติกสายพันธุ์ที่ลดคอเลสเตอรอลเพื่อป้องกันภาวะเมตาบอลิกซินโดรม

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Received: February 28, 2023

Revised: March 20, 2023

Accepted: April 11, 2023

บทคัดย่อ

เมตาบอลิกซินโดรมเป็นกลุ่มอาการผิดปกติของการเผาผลาญที่มีปัจจัยมาจากการมีภาวะน้ำหนักเกิน ทานอาหารแคลอรีสูง ออกกำลังกายน้อย ทำให้เกิดโรคอ้วน ไขมันในเลือดสูง ความดันโลหิตสูง น้ำตาลในเลือดสูง นำไปสู่การเกิดโรคไม่ติดต่อเรื้อรัง มีหลายรายงานแสดงถึงการได้รับโพรไบโอติกในปริมาณที่เหมาะสมสามารถลดปัจจัยเสี่ยงของเมตาบอลิกซินโดรมได้ การศึกษานี้คัดเลือกโพรไบโอติกสายพันธุ์ที่สามารถลดคอเลสเตอรอลลดการอักเสบ และเพิ่มสารต้านอนุมูลอิสระ ได้แก่ *Lactobacillus paracasei* MSMC39-1, *Lactobacillus reuteri* TF-7 และ *Bifidobacterium animalis* MSMC83 มาศึกษาสัณฐานวิทยาของเซลล์ วิเคราะห์อัตราการเจริญเติบโตและการสร้างโพรไบโอติก ซึ่งพบว่าโพรไบโอติกสามารถผลิตโพรไบโอติกชนิดกรดโพรพิโอนิกและกรดบิวทีริกได้ จากนั้นนำโพรไบโอติกมาเลี้ยงในถังหมักชีวภาพขนาด 5 ลิตรเพื่อหาสภาวะที่เหมาะสมในการเพิ่มผลผลิต โพรไบโอติกในรูปแบบผงแห้งทั้ง 3 สายพันธุ์บรรจุลงในแคปซูลในปริมาณความเข้มข้นเท่ากัน ทดสอบการรอดชีวิตในทางเดินอาหารจำลอง ซึ่งพบว่าโพรไบโอติกในแคปซูลสามารถรอดชีวิตได้อย่างมีนัยสำคัญเมื่อเทียบกับเซลล์อิสระ และสามารถรอดชีวิตได้ระหว่างการเก็บรักษาเป็นเวลา 6 เดือน ที่อุณหภูมิ 4 และ 25 องศาเซลเซียส โดยมีปริมาณคงเหลือมากกว่า 10^6 CFU ต่อกรัม อีกทั้งยังคงคุณสมบัติในการลดคอเลสเตอรอลโดยโพรไบโอติกในแคปซูลสามารถสร้างเอนไซม์ไลโซลทิไฮโดรเลสได้ตลอดการเก็บรักษาลักษณะ ดังนั้นผลิตภัณฑ์โพรไบโอติกแคปซูลสามารถนำไปประยุกต์ใช้ในผู้ที่มีปัจจัยเสี่ยงของภาวะเมตาบอลิกซินโดรมได้ อย่างไรก็ตาม จำเป็นต้องมีการศึกษาเพิ่มเติมในอาสาสมัครเพื่อประเมินประสิทธิภาพของผลิตภัณฑ์โพรไบโอติก

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Characterization and product formulation of cholesterol-lowering probiotic strains for prevention of metabolic syndrome

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Abstract

Metabolic syndrome (Mets) is influenced by factors such as being overweight, consuming a high calorie diet, and little exercise. Obesity, dyslipidemia, hypertension, and hyperglycemia are all part of Mets, which may result in non-communicable diseases. Several researches indicated that moderate consumption of certain probiotic strains could reduce the risk factors of Mets. In this study, three probiotic strains including *Lactobacillus paracasei* MSMC39-1, *Lactobacillus reuteri* TF-7 and *Bifidobacterium animalis* MSMC83 with specific properties in lowering cholesterol, reducing inflammation, and increasing antioxidants, were selected. Cell morphological phenotypes of each probiotic were studied by a scanning electron microscope. The growth characteristics of postbiotics were analyzed and probiotics were cultivated in a 5-liter bioreactor to optimize the best conditions to increase their yield. A combined capsule product was formulated with probiotic ingredients. These products were evaluated for tolerance to simulated gastrointestinal conditions, viability and stability at 4°C and 25°C, and ability to produce bile salt hydrolase (BSH). The results indicated that postbiotics propionic acid and butyric acid were fermented by probiotic metabolites. Probiotic products significantly increased probiotic survival when compared with probiotic-free cells in simulated gastrointestinal conditions. In addition, the viability of probiotics was demonstrated at concentrations greater than 10⁶ CFU/g after storage at 4°C and 25°C for 6 months. Cholesterol-lowering properties of BSH of the probiotics in capsule were maintained during storage. Therefore, the probiotic product can be applied to improve anthropometric and biochemical outcomes in individuals with MetS. However, more research is needed to evaluate the effectiveness of probiotic products in clinical trials.

Keywords: probiotics, *Lactobacillus*, *Bifidobacterium*, metabolic syndrome

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Introduction

Metabolic syndrome (MetS) is a cluster of metabolic diseases that includes obesity, decreased glucose tolerance, insulin resistance, elevated blood pressure, and dyslipidemia. The prevalence of MetS is correlated with obesity, poor diet, inactivity, and genetic disorders. In addition, MetS plays a significant role in the increased risk of non-communicable diseases (NCDs), including type II diabetic mellitus (T2DM), cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD)¹. Long-term medication or traditional therapy has a variety of disadvantages. Therefore, researchers currently study how to use microbes to treat and prevent diseases in humans. The microorganisms which benefit the host through symbiosis are known as probiotics. Previous studies showed that administration of *Lactobacillus plantarum* and *Bifidobacterium breve* in overweight patients for 12 weeks increased high-density lipids with lower waist circumference and reduced lipid profile, glucose levels, triglycerides and cholesterol levels in blood².

In 2014, the definition of probiotics was defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”³. Probiotics can be found in the gastrointestinal tract as normal microbiota. Each person has a variety of probiotic species and quantities according to their genetic makeup, age, lifestyle, and nutrition. Probiotic strains are furthermore

unique to individuals of various races⁴. Moreover, sources of probiotics include dairy products, fermented foods, fruits, and vegetables⁵. There are several genera of probiotics that are commercially available. The most popular to utilize in probiotic supplements are *Lactobacillus* and *Bifidobacterium*. Probiotics have a variety of properties that vary depending on the strain, such as reducing blood sugar and cholesterol levels⁶, and body mass index⁷, improving the immune system, anti-inflammatory⁸ and antioxidant⁹.

Several mechanisms have been proposed for elimination of cholesterol by probiotics, such as probiotics are able to bind with free cholesterol in the intestinal lumen leading to reduction of cholesterol absorption and formation of cell membrane¹⁰. Probiotics are able to produce bile salt hydrolase enzyme (BSH), which deconjugates bile salts and brings cholesterol in the liver to produce bile replacement¹¹. Additionally, short-chain fatty acids (butyrate, propionate, and acetate), which are postbiotic substances produced or released by probiotic metabolic activities, are also important for anti-inflammatory, immunomodulatory, and antioxidant activities, modulation of the gut microbiota, regulation of obesity, and glucose homeostasis^{12,13}.

The global trend toward consuming healthy food items is now growing substantially. Healthy foods, dietary supplements, and functional foods are becoming as alternatives and strategies for promoting health to reduce risks and prevent diseases. Healthy food

inventions termed probiotic products are becoming more and more well-liked on a worldwide scale. The industry has been adding a range of probiotic into products as a result of the rising demand for probiotic-containing goods and enhanced the value, benefits, and selling factors of healthy food items. The majority of probiotic supplements are dairy-based and come in powder, tablet, and capsule forms. In particular, viability and stability of probiotics in the product must be taken into account because the probiotic microbes are live microorganisms. To meet Thai FDA requirements, a product's end-of-shelf life must contain at least 10^6 CFU/g of survival probiotics¹⁴. The probiotics used in the product must withstand environmental conditions such as the processing of raw materials, the creation of finished goods, storage, and transportation. Temperature, pH, relative humidity, oxygen levels, product composition, and packaging are some factors that have an impact on probiotics' ability to survive¹⁵. Moreover, additional ingredients including flavors, colors, various extracts and other microorganisms also have an impact on probiotics in the product during storage. In order to improve probiotics' survival, prebiotics are also added to the product. Therefore, probiotic strains, encapsulation technology and packaging as well as creating synbiotic are important steps of product generation. Additionally, the probiotic products must also be resistant to the gastrointestinal conditions. A previous study found that of 11 commercial probiotic samples, only two had the same

concentration as indicated on the product label¹⁶. Therefore, maintaining product quality requirements must be considered while developing probiotic products in order to guarantee that the probiotic content and specific properties last during the shelf life. For the best interest of customers, one must concentrate on the research and development of innovative products.

Previous studies indicated that Thai probiotic strains reduced plasma lipid and glucose levels, decreased lipid accumulation in the liver, and reduced the inflammatory response in animal models. *In vitro* and *in vivo* studies confirmed that *L. reuteri* TF-7 and *B. animalis* MSMC83 decreased blood sugar via the glycolysis pathway and directly lowering the cholesterol level via the activity of the bile salt hydrolase enzyme^{17,18}. In addition, *B. animalis* MSMC83 also increased levels of antioxidant enzymes leading to reduction of free radical levels in blood and liver of D-galactose induced oxidative stress model^{18,19}. Moreover, inflammation and level of TNF-alpha in livers of alcohol-induced hepatitis rat model were decreased with *L. paracasei* MSMC39-1 administration²⁰. From these studies, we selected *L. reuteri* TF-7, *L. paracasei* MSMC39-1 and *B. animalis* MSMC83 for our study. Therefore, the objective of this study was to produce a combined probiotic product for the treatment and prevention of the metabolic syndrome. The morphology, growth rate and postbiotic production were studied. The probiotic ingredients were produced in capsule form. During storage,

viability, stability, and bile salts hydrolase activity of probiotic product were measured at different temperatures, and tolerance to simulated gastrointestinal conditions was also evaluated.

Materials and Methods

Preparation of probiotics and growth conditions

Probiotic strains *L. paracasei* MSMC39-1, *L. reuteri* TF-7, and *B. animalis* MSMC83 were selected in this study. Three strains of probiotics isolated from Thai food and Thai infants were obtained from Center of Excellent in Probiotics, Faculty of Medicine, Srinakharinwirot University (Bangkok, Thailand). The safety of all probiotic strains was previously confirmed *in vitro* and in animal models. Additionally, all probiotic strains were listed in the Ministry of Public Health's announcement that they can be used as probiotic microorganisms in food by the Thai FDA.

All probiotic strains were maintained as frozen (-80 °C) stocks and cultured on de Man Rogosa Sharp (MRS; HiMedia, India) broth, incubated at 37 °C for 24-48 hours under anaerobic conditions (10% O₂, 10% CO₂, 80% N₂) using the anaerobic jar (Mitsubishi, Japan). Then, the selected probiotic strains were sub-cultured on MRS agar three times before the experiment.

Morphological analysis by using a scanning electron microscope

The probiotics' morphology was examined using a scanning electron microscope

(Hitachi High-Technologies, Japan). Colony of probiotic samples were prepared on a stub using carbon tape and coated in gold particles to facilitate electrical conductivity. The condition of an electron in a vacuum were set at the electric potential acceleration of 20 kV.

Growth characteristics of selected probiotic strains

The selected probiotic strains were cultured in MRS broth and incubated at 37 °C for 36-62 hours under anaerobic conditions. Probiotics at a concentration of 10⁸ colony forming units (CFU)/mL were inoculated into MRS broth. Then, the free cells culture growth was investigated by measuring optical density (OD) every 2 hours (n=3).

Measurement of postbiotic production by high performance liquid chromatography (HPLC)

Probiotics were cultured in MRS broth and incubated at 37 °C for 48 hours for *Lactobacillus* and 72 hours for *Bifidobacterium* under anaerobic conditions. The obtained solution from the probiotics culture was centrifuged (4,000 g, 4 °C, 10 min) and filtered with a sterilized syringe filter of 0.22 µm to separate bacterial cells from postbiotic metabolites. One N of NaOH was used to adjust the pH of the postbiotic solution to 6.5-7.0. Then the solution was filtered and aliquots into a 2 mL vial. 20 µL of samples were injected into column Biorad Aminex-87H and analyzed by HPLC (Shimadzu LC-10AD, Corporation, Japan), using mobile phase 5 mM

sulfuric acid, flow rate 0.6 mL/min and column temperature of 50 °C. Propionic acid and butyric acid of postbiotics were detected by UV detectors.

Probiotic scale-up productions

For optimum conditions of culturing, the temperature was controlled at 37 °C in the bioreactor (5L) of each probiotic strain. The pH was controlled between 6.0-6.2 with a stirring rate of 150 rpm and a Feeding N₂ at flow rate of 0.02 vessel volumes per minute (VVM). *Lactobacillus* cells and *Bifidobacterium* cells were harvested at the end of 18 and 24 hours, respectively.

Probiotic capsule preparation

Probiotic ingredients of *L. paracasei* MSMC39-1, *L. reuteri* TF-7, and *B. animalis* MSMC83 were formulated and developed at Center of Excellence in Probiotics, Srinakharinwirot University (Bangkok, Thailand). The finish probiotic product was packaged at a factory (Adsgo Drugs Co., Ltd., Thailand) certified by GMP standards according to the standards of the Thai FDA. The probiotic ingredients at the same concentration were packaged in hydroxypropyl methylcellulose (HPMC) capsules (Lonza's DRcaps®). The final concentration was 10¹⁰ CFU/capsule. Resistant maltodextrin and silicon dioxide were added to prevent moisture in the product. Probiotic capsules were packaged in aluminum foil to protect them from light.

Tolerance to simulated gastrointestinal tract of probiotics in capsule products

The probiotic capsules were placed in simulated gastric juice at pH 3.0 containing 0.35% (w/v) of pepsin (HiMedia, India) and 0.2% (w/v) NaCl (Merck, Germany) in deionized water and incubated at 37 °C. After 3 hours incubation, the capsules were re-placed in simulated intestinal juice containing 1.8% (w/v) of bovine bile (Sigma-Aldrich, USA), 0.1% (w/v) of trypsin (HiMedia, India), 1.1% (w/v) of NaHCO₃ (Merck, Germany) 0.2% (w/v) NaCl (Merck, Germany), and incubated at 37 °C for 3 hours. The viable cells were evaluated by spread plate method on MRS agar at 37 °C for 24-48 hours. The free cells of each probiotic ingredient were used as controls. Cell viability was expressed as log CFU/g.

Viability of probiotics in capsule product during storage

The probiotic capsules were stored at 4 °C and 25 °C for 6 months. Every month, the product was evaluated for the viability and stability of probiotics by dissolving the capsules in phosphate-buffered solution (PBS; pH 7.2) buffer, and 10-fold serial dilution was made. After that, the suspension was spread on MRS agar and incubated at 37 °C for 24-48 hours under anaerobic conditions. Viable probiotic cells were counted and reported as CFU/g.

Bile salt hydrolase (BSH) activity test in products during storage

Every month, the probiotic product was dissolved in PBS buffer and dripped onto MRS agar supplemented with 0.5% (w/v) sodium taurodeoxycholic acid (TDCA; Sigma, USA) and 0.37% CaCl_2 (Merck, Germany), then incubated at 37 °C in anaerobic conditions for 72 hours. Production of BSH enzymes was observed, and the results were interpreted according to the size of bile precipitation zones.

Statistical analysis

All experiments were performed three times independently. The results were expressed as means $\pm$ standard deviation (SD). Statistically significant analysis of data was

performed by one-way analysis of variance and a student's t-test using GraphPad Prism software (GraphPad software, version 8.0.1, San Diego, CA, USA). A p-value less than 0.05 was considered significant.

Results

Morphological characteristics of probiotics by scanning electron microscopy

The morphologies of *L. paracasei* MSMC39-1, *L. reuteri* TF-7, and *B. animalis* MSMC83 were shown in figure 1A-C. *L. paracasei* MSMC39-1 cells (Figure 1A) appeared as slender rods, whereas *L. reuteri* TF-7 cells (Figure 1B) were short rod-shaped. *B. animalis* MSMC83 cells morphology was dumbbell and club-shaped bacteria (Figure 1C).

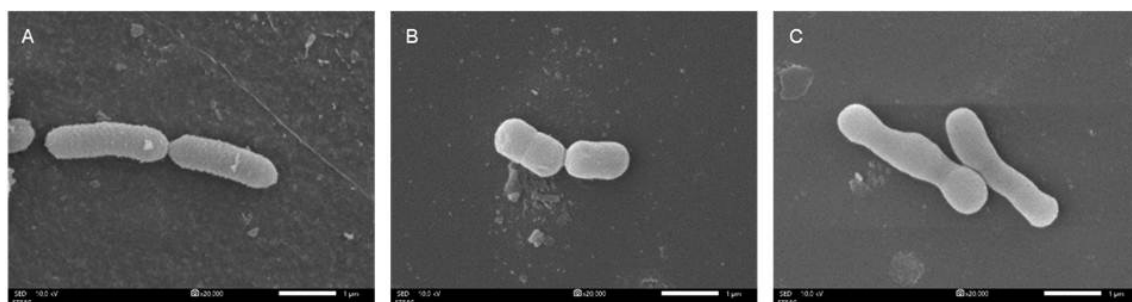


Figure 1 The scanning electron microscope (SEM) images of selected strains: (A) *Lactobacillus paracasei* MSMC39-1, (B) *Lactobacillus reuteri* TF-7, and (C) *Bifidobacterium animalis* MSMC83. All micro images showed 20,000x magnifications.

Growth characteristics of probiotics

Each probiotic strain was inoculated into MRS broth, and continuously cultured at 37 °C for 36-62 hours, and their growth profiles were investigated using OD measurements.

After 24 hours, *L. paracasei* MSMC39-1 entered log phase growth. Then, maintained at a stationary phase after 30 hours cultured (Figure 2A). *L. reuteri* TF-7 entered log phase growth after 12 hours and maintained stationary phase

at 22 hours after cultured (Figure 2B). Additionally, *B. animalis* MSMC83 had the longest exponential growth phase, entered

log phase growth after 36 hours and maintained stationary phase at 62 hours after cultured (Figure 2C).

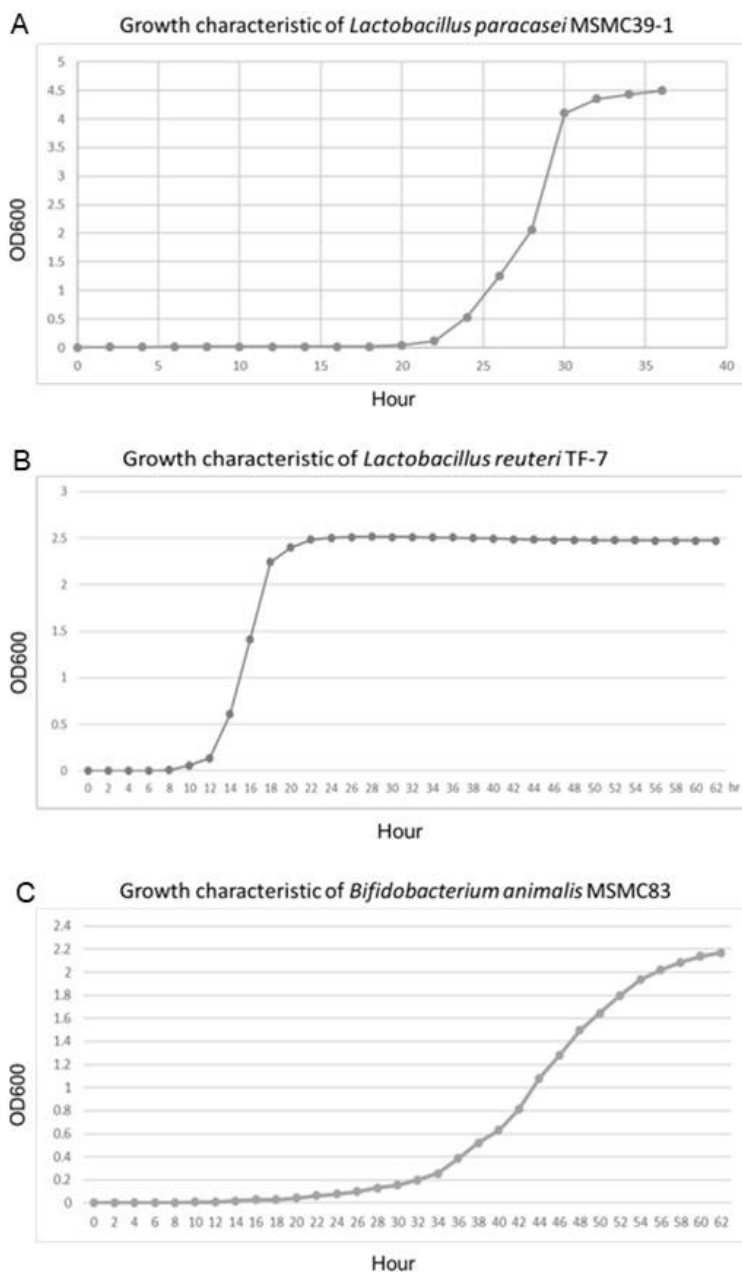


Figure 2 Growth curves of probiotics selected strains: (A) *Lactobacillus paracasei* MSMC39-1, (B) *Lactobacillus reuteri* TF-7, and (C) *Bifidobacterium animalis* MSMC83. Cell density was measured every 2 h (n = 3).

Production of postbiotics

The production of propionic acid and butyric acid of fermented samples including *L. paracasei* MSMC39-1, *L. reuteri* TF-7 (after 48 hours) and *B. animalis* MSMC83 (after 72 hours) in MRS broth at 37 °C were obtained from HPLC chromatograms. As shown in Table 1, at the end of fermentation process, 0.536%

(w/v), 0.723% (w/v), and 0.491% (w/v) of propionic acid were produced from *L. paracasei* MSMC39-1, *L. reuteri* TF-7, and *B. animalis* MSMC83, respectively. In addition, generations of butyric acid were as followed, 0.187% (w/v) for *L. paracasei* MSMC39-1, 0.209% (w/v) for *L. reuteri* TF-7, and 0.385% (w/v) for *B. animalis* MSMC83.

Table 1 HPLC Chromatograms from fermented samples of *L. paracasei* MSMC39-1, *L. reuteri* TF-7 and *B. animalis* MSMC83

Samples	Propionic acid (%w/v)	Butyric acid (%w/v)
<i>L. paracasei</i> MSMC39-1	0.536	0.187
<i>L. reuteri</i> TF-7	0.723	0.209
<i>B. animalis</i> MSMC83	0.491	0.385
MRS Broth	Not detected	Not detected

Viability of probiotics in capsule products with simulated gastrointestinal tract condition

All samples were incubated for 6 hours in simulated gastric and intestinal juices to examine survivability of probiotics mixed in capsules and free cells. The viability of probiotics was expressed as log CFU/mL. The results of *in vitro* gastrointestinal tract tolerance assay were shown in Table 2. After 3 hours of exposure to simulated gastric juice, free cells of *L. paracasei* MSMC39-1, *B. animalis* MSMC83, and *L. reuteri* TF-7 showed significantly decreased in cell viability ($p<0.05$) with the lowest reduction of 3.20 ± 0.02 , 5.45 ± 0.05 ,

and 7.36 ± 0.14 log CFU/mL, respectively. While the viability of probiotics combined in capsule was not significantly decreased when compared with initiation. However, the viability of free cells of *L. paracasei* MSMC39-1, and *L. reuteri* TF-7 was not significantly decreased ($p<0.05$) during 3 hours of exposure to simulated intestinal juice when compared with simulated gastric juice. While the viability of probiotics in capsules and free cells of *B. animalis* MSMC83 was significantly reduced ($p<0.05$) and decreased to 7.93 ± 0.15 , and 4.64 ± 0.04 log CFU/mL, respectively (Figure 3).

Table 2 Viability of probiotics in the digestion of simulated gastrointestinal tract

Probiotics	Number of viability probiotics (log CFU/mL)		
	Initiation (0 hour)	Simulated gastric juice	Simulated intestinal juice
Probiotics capsule	10.85 ± 0.12	10.74 ± 0.03	7.93 ± 0.15 ^{##}
<i>L. paracasei</i> MSMC39-1	10.71 ± 0.09	3.20 ± 0.02 ^{***}	2.88 ± 0.15
<i>L. reuteri</i> TF-7	10.57 ± 0.06	7.36 ± 0.14 ^{**}	6.90 ± 0.06
<i>B. animalis</i> MSMC83	10.45 ± 0.06	5.45 ± 0.05 ^{**}	4.64 ± 0.04 [#]

^{**} $p < 0.01$, ^{***} $p < 0.001$ as compared with initiation, [#] $p < 0.05$, ^{##} $p < 0.01$ as compared with simulated gastric juice.

Viability of probiotics in capsule products during storage

The initial viability counts of probiotics combined in the capsule were 10.67 ± 0.06 log CFU/g for storage at 4 °C and 10.55 ± 0.00 log CFU/g for storage at 25 °C. The viability of probiotics capsules stored at 4 °C slightly decreased the viability to 9.842 ± 0.05 log

CFU/g at the end of the storage period (6 months). On the other hand, storage at 25 °C was significantly reduced to 8.147 ± 0.05 log CFU/g after 3 months of storage and 7.425 ± 0.17 log CFU/g at the end of shelf-life (6 months) (Figure 3). Therefore, low storage temperature (4 °C) helps to maintain probiotics viability.

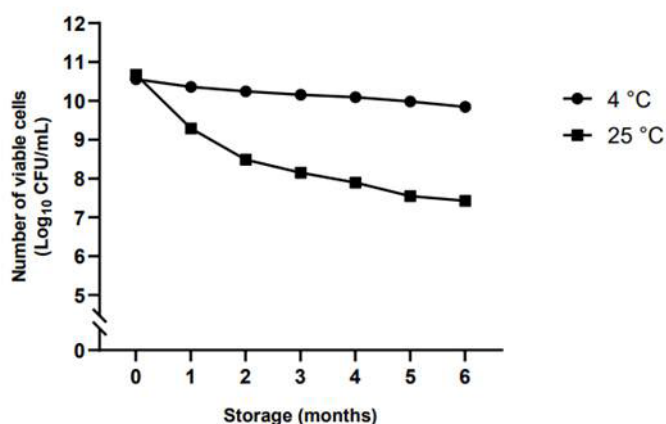


Figure 3 The viability of probiotics in capsule products during storage at 4 °C and 25 °C

The stability of bile salt hydrolase (BSH)

The stability of bile salt hydrolase (BSH) property of probiotics was studied by qualitative direct plate assay, and taurodeoxycholic acid (TDCA) was used as substrate. Probiotics in

capsules retained stability of BSH enzymatic properties and showed potency along the size of bile precipitation zones as compared to unprocessed probiotics (Table 3, Figure 4).

Table 3 The stability of the bile salt hydrolase (BSH) enzymatic properties of probiotics during storage

Probiotics	Storage (Month)						
	0	1	2	3	4	5	6
<i>L. reuteri</i> TF-7 free cell	+++	+++	+++	+++	+++	+++	+++
<i>B. animalis</i> MSMC83 free cell	++	++	++	++	++	++	++
Probiotic capsule	+++	+++	+++	+++	+++	+++	+++

The bile salt hydrolase (BSH) enzyme was measured according to the diameter of colony precipitation on TDCA agar: - ; Probiotic

did not grow, ++ ; ≤ 8.0 mm, +++; ≤ 8.1-13.0 mm.

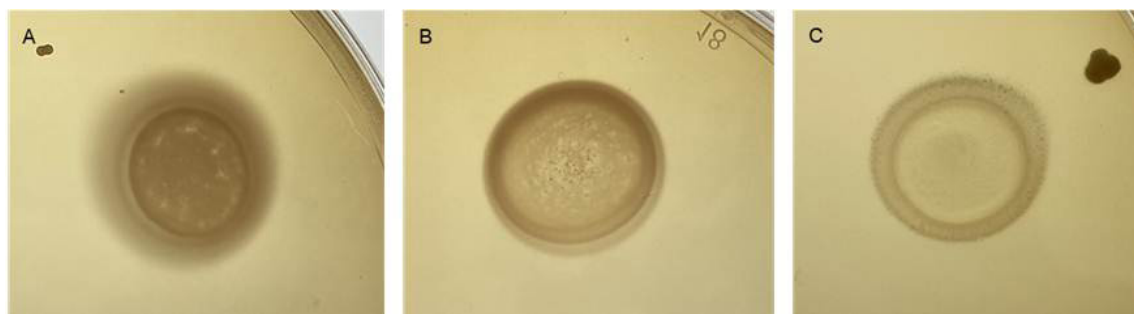


Figure 4 Bile salt hydrolase (BSH) activity: (A) *Lactobacillus reuteri* TF-7, (B) *Bifidobacterium animalis* MSMS83 and (C) Probiotic capsules

Discussion

Beneficial gut microbiota using substrates to support human metabolism has recently attracted a lot of attention. Moreover, several studies have demonstrated that probiotics can improve the immune system and the gut microbiota, which can improve metabolic syndrome parameters. In this study, we used *Lactobacillus paracasei* MSMC39-1, *Lactobacillus reuteri* TF-7, and *Bifidobacterium animalis* MSMC83 to formulate a combination probiotic product for treatment and prevention of metabolic syndrome.

We studied the morphological phenotypic of probiotic strains by scanning electron microscopy and analyzed their growth characteristics to optimize conditions to increase yield. Probiotics were cultured in MRS medium, which is a rich medium with a mixture of different carbon sources and complex nitrogen sources. Besides, conditions like temperature, pH, oxygen concentration, or water activity, among others, have an impact on the growth of probiotics²¹. The optimum temperature and pH conditions for *Lactobacillus* growth are between 30-40 °C

and pH 5.5-6.2²². In addition, the optimum temperature and pH for the growth of *Bifidobacterium* are between 36-38 °C and 6.5-7.0, respectively²³. In this study, the pH was controlled between 6.0-6.2, which was optimal for the growth of the three probiotic strains. The exponential growth phase was observed in *L. paracasei* MSMC39-1 and *L. reuteri* TF-7 after 12-24 hours incubation. Whereas *B. animalis* MSMC83 had the longest period of an exponential growth phase which was observed after 36 hours of incubation.

In 2021, the ISAPP defined a postbiotic as “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”²⁴. Postbiotics are metabolites secreted by probiotics, including short-chain fatty acids (SCFAs), exopolysaccharides, enzymes, and vitamins²⁵. The propionic acid and butyric acid as SCFAs are produced during the colonic microbiota’s fermentation of dietary fibers. These SCFAs have been described as having anti-obesity properties by improved fasting glycemia, body weight, and insulin tolerance²⁶. In addition, the SCFAs have an impact on rat primary adipocyte glucose absorption, fat storage, and mobilization. Accordingly, the SCFAs may support better adipocytes, which may then lead to improved energy metabolism, such as fewer circulating free fatty acids, which is advantageous in obesity and type 2 diabetes²⁷. Type 2 Diabetes (T2D) is associated with composition and functional changes in the gut microbiota²⁸. Interestingly, the abundance of butyrate-producing bacteria and the terminal genes for

butyrate synthesis were reduced in T2D and prediabetes²⁹. Propionic acid increases gluconeogenesis by increasing the hepatic turnover of pyruvate through conversion to succinate, a potent stimulator of endogenous glucose production in various mammals³⁰. Correlation between the levels of butyrate and propionic acid and metabolic parameters was also investigated in individuals with T2D³¹. Therefore, all probiotic strains in this study can produce propionic and butyric acids, which may contribute to improving the effects of metabolism.

At present, probiotic supplements have gained more attention for health promotion. To obtain health benefits, probiotics must be able to survive in the gastrointestinal tract. Moreover, probiotics must also maintain their viability throughout storage until the end of their shelf life. The consumption of probiotics is put through a number of stressful situations in the digestive system, in particular, the fluctuation of human digestive system’s pH. This ranges from pH 6-7 in the mouth, pH 1-3 in the stomach, and pH 6-7 in the small and large intestines³². This is brought on by low stomach acid pH and high small intestine bile salt concentration³³. Therefore, maintaining the viability of the probiotic microorganisms that reach the colon is important. Three probiotic strains in HPMC capsule were highly resistant to gastric juice and intestinal gastric juice than free-cell probiotics. Besides, the viability remained sufficient to meet the recommendations of the FDA. The recommended minimum

number of probiotics in food products is at approximately 10^6 CFU/g. HPMC capsules protect sensitive ingredients from acidic environment of the stomach and dissolve completely in the intestines³⁴. The ability of probiotic strains to survive in the gastrointestinal system is crucial to their effectiveness. High doses of probiotics might enhance the likelihood of colonization in the gut since certain probiotics may have a poor survival rate. Moreover, temperature and moisture content are important factors to consider during storage. Probiotic capsules were packaged in foil pouches per capsule to eliminate the risk factor for loss of probiotics from moisture after opening the package. The viability of the probiotic capsules was monitored during storage at each temperature throughout the research period. After 6 months, the number of probiotics slightly decreased when stored at 4 °C. However probiotic content was greatly reduced when stored at 25 °C. Therefore, it is necessary to store probiotic products at temperatures below or equal to 25 °C and lower moisture for longer shelf life.

Several studies also examined the effectiveness of probiotics to reduce cholesterol level. The effects of probiotics on hypercholesterolemia depend on the dosage, frequency, and duration of consumption for the different probiotic strains. Our previous research in animal models found that *L. reuteri* TF-7 and *B. animalis* MSMC83 decreased cholesterol levels in rats fed with a high-fat diet. The selected probiotic strains *L. reuteri*

TF-7 and *B. animalis* MSMC83 were able to produce BSH^{17,18}. Several mechanisms of probiotics have been reported to lower cholesterol levels. Probiotics can bind to cholesterol to build cell membranes and reduce the amount of cholesterol in the circulation³⁵. Cholesterol is incorporated into cell membranes of bacteria to increase cell membrane strength and resistance to breakdown³⁶. Probiotics can also convert cholesterol into coprostanol in the intestines by cholesterol-lowering bacteria and are excreted directly in the feces. Thus, the amount of cholesterol absorbed is reduced and resulting in a decreased in cholesterol accumulation in the body decreased³⁷. The indirect mechanism of cholesterol-lowering probiotics is BSH activity. Probiotic-produced BSH catalyzes the deconjugation of bile salts. The hydrophobic deconjugated bile salts are reabsorbed less readily in the intestine, resulting in higher excretion in feces. Therefore, the amount of bile salts that are returned to the liver and circulated between the liver and intestines are reduced. As a result, serum cholesterol is reduced, and lost bile salts are replaced by increased de novo synthesis³⁸⁻⁴⁰. In this study, selected probiotic strains *L. reuteri* TF-7 and *B. animalis* MSMC83 were still able to produce BSH when examining the stability of BSH activity after manufacture and during storage. Therefore, it is reasonable to assume that these probiotics in products have the ability to lower cholesterol and could possibly be used in the prevention of hypercholesterolemia.

Conclusion

Three probiotic strains that were developed into capsule formulations were viable and stable. The probiotics in capsules were able to survive and maintain their cell viability at the concentration greater than 10^6 CFU/g when stored at 4 °C and 25 °C. The products in capsule form can help maintain the survival rate of probiotics in a simulated gastrointestinal tract condition. The cholesterol-lowering properties of bile salt hydrolase were maintained throughout storage. Therefore, the product in this research will improve stability and increase product value, as well as encourage reliance on already-available technology and creation of innovation. Additionally, probiotic products could be used in individuals with MetS, and further efficacy in clinical trials is needed.

Acknowledgements

The study was supported by the Faculty of Medicine and HRH Princess Maha Chakri Sirindhorn Medical Center, Srinakharinwirot University (Grant number 079/2565), and supported by Center of Excellence in Probiotics, Srinakharinwirot University (Grant number 324/2565).

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