CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Media and chemical reagents

List of media and chemicals	Production companies
Acetone	Merck, Germany
Agar	-
Agarose	-
Antibiotics	Sigma, Germany
-Ampicillin	
-chloramphenicol	
-Ciprofloxin	
-Quinupristin/dalfopristin	
-Erythromycin	
-Gentamicin	
-Kanamycin	
-Linezolid	
-Rifampicin	
-Streptomycin	
-Tetracycline	
-Trimetroprim	

List of media and chemicals	Production companies
Apple pectin	Roth, Austria
Di-ammonium hydrogen citrate	Merck, Germany
Brain Heart Infusion broth	Merck, Germany
Crystal violet	Merck, Germany
Disodium hydrogen phosphate	Merck, Germany
Ethanol 95%	-
D-Glucose	Merck, Germany
dNTP-Mix	Roth
Glycerol	Fisher
Hydrochloric acid 37%	Merck; Germany
3% hydrogen peroxide	Witthayasom, Thailand
Hydroxy methyl cellulose (Metolose®)	Shin-Etsu chemicals Ltd., Japan
Hydroxymethyl cellulosephthalate (HPMCP 55)	Shin-Etsu chemicals Ltd., Japan
Inulin	Fluka, Germany
Iodine	-
Lactose monohydrate	Kwizda, Austria
Magnesium stearate	Kwizda, Austria
Magnesium sulphate	Fluka
Manganese sulphate	UNIVAR
Meat extract	Merck, Germany
MRS (De Man, Rogosa and Sharpe) broth	Merck, Germany
MRS agar	Merck, Germany

List of media and chemicals	Production companies
Mueller Hinton Agar	Merck, Germany
n-hexadecane	Sigma, Germany
Oxgall	Difco, USA
Peptone	Merck, Germany
Dipotassium hydrogen phosphate	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Potassium iodide	M&D Laboratory Chemicals
Raftiline®(commercial grade inulin)	Orafti Active Food Ingredients
Safranin	Merck, Germany
Skimmed milk	Merck, Germany
Sodium acetate	Merck, Germany
Sodium alginate	Buchs, Switzerland
Sodium chloride	Merck, Germany
Sodium glutamate	Fluka
Sodium hydroxide	Merck, Germany
Sucrose	-
Talcum	Kwizda, Austria
Tween 80	Merck, Germany
Yeast extract	Merck, Germany

3.1.2 Kits

- API CH 50 (Biomerieux, France)
- Biolog AN MicroPlate (Biolog, Inc., Hayward, CA, U.S.A.)

3.1.3 Primers

: Lac-2, Ldel-7, LU-5, LU-3, LU-1, LbLMA1/R16, ReuI/ReuII, Lferm 3/Lferm 4, Lpla 2/ Lpla3

3.1.4 Equipments

List of equipments	Production companies
Analytical blance, Sartorious CE95	Sartorious, Germany
Analytical blance, Metler Toledo PG-802-S	Germany
Autoclave	Edusystem
Centrifuge Harrier	Sanyo, Japan
Cube mixer	Erweka-Apparatebau GmbH, Germany
Deep freezer	Liebherr, Germany
Disintegration apparatus PTZ1	PharmaTest GmbH, Germany
Dissolution tester Type PTWS3C	PharmaTest GmbH, Germany
Freeze dryer Christ1-4	Christ GmbH, Germany
Hardness tester PTB-311	PharmaTest GmbH, Germany
Hot air oven	Binder
Hot air oven	Memmert, Germany
Incubator Memmert Type UE500	Memmert, Germany
Incubator	Binder
Laminar air flow Cabinet Class II	Microflow
Microscope	Olympus
pH meter pH 539	WTW, Germany

List of equipments	Production companies
pH meter	Metrohm, Swiszerland
Single punch tablet machine	Korsch EKO, Germany
Spectrophotometer U-3000	Hitachi, Japan
Spectrophotometer 20	Genesys, USA
UV/VIS Spectrophotometer	Perkin-Elmer, USA
Water bath	Memmert, Germany

3.1.5 Animals

Male Swiss albino mice with the age of 5 weeks old and 30-35 g body weight obtained from the National Laboratory Animals Center, Mahidol University, Nakhonpathom province, Thailand were used in this study.

3.1.6 Samples

Lactic acid bacteria (LAB) were isolated from one hundred faecal samples of healthy children obtained from kindergarten and individual contact in Chiang Mai Province, northern of Thailand. One hundred samples of traditionally Thai fermented foods purchased from local markets in Chiang Mai area were also used as sources of LAB.

3.1.7 Bacterial indicators

- *Escherichia coli* TISTR 780
- *Salmonella typhi* DMST 5784
- *Staphylococcus aureus* TISTR 029

- *Lactobacillus delbruckii* ssp.*bulgaricus* TISTR 892

- *Lactobacillus acidophilus* La5 (Chr.hansen Biosystems, Horsholm, Denmark)

- *Lactobacillus acidophilus* 72-4 (Chr.hansen Biosystems, Horsholm, Denmark)

3.2 Methods

3.2.1 Bacterial strains and Isolation

Lactic acid bacteria (LAB) were isolated from one hundred faecal samples of healthy children obtained from kindergarten and individual contact in Chiang Mai Province, northern of Thailand. One hundred samples of traditionally Thai fermented food products purchased from the food galleries local markets in Chiang Mai area were used as sources of LAB.

1) Isolation of LAB from feces samples

Small amount of faeces were collected in sterile small bottle and added MRS broth as carry medium. Then, feces samples were streaked on MRS agar plates and incubated in microaerobic atmosphere for 48 hours at 37 ^{o}C.

2) Isolation of LAB from fermented foods

The sample was mixed with normal saline to appropriate dilutions. A volume of 0.1 ml of the dilutions was plated on MRS agar and incubated in anaerobic condition at 37 °C for 48 h.

The typical isolated colonies were taken randomly for purification. The purified colonies were tested for lactobacilli by microscopic examination with gram stain and catalase production. The gram positive rods with catalase-negative strains

were selected for further studies. All of themwere maintained as frozen cultures in MRS broth containing 30% glycerol at –20 ^{o}C.

The isolates from children faeces and fermented foods were investigated for probiotic characterization, comprising resistance to acid and bile tolerance as screening tests.

3.2.2 Screening of acid and bile tolerant isolates

The isolated lactobacilli were firstly tested for acid tolerance screening by growing in the MRS broth adjusted to pH 2.5 with 1N HCl. The survival was determined by single streaking of this culture broth after 90 min at 37 °C incubation on MRS agar plates. The growth was observed after 24-48 h after anaerobic incubation at 37 °C. Isolates demonstrated the growth on the agar were considered to be acid tolerant. The screening for bile tolerance was carried out by growing in MRS broth containing 0.3% of Oxgall® bile salt for 24 h of anaerobic incubation at 37 °C. The culture broth with turbidity more than 0.5 units at 600 nm was classified as bile tolerant strains.

3.2.3 Determination of acid resistant

In this study, only the acid and bile tolerant strains were used. The strains were cultivated at 37 °C in MRS broth under an anaerobic atmosphere. A portion of 10^{7}–10^{8} cfu/ml culture was inoculated in 10 ml of 0.05 M sodium phosphate buffer. This buffer was prior adjusted to pH 2.0, 2.5, 3.0, and 7.0 with 1 N HCl. After incubation at 37 °C for 2 h, cells were serially adjusted to 10-fold dilution by phosphate buffer pH 7.0. The dilution was plated on MRS agar for determination of viable cells after

48 h of incubation period. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration. The experiment was performed in triplicate.

3.2.4 Determination of bile resistant.

The overnight MRS broth cultures with 10^7–10^8 cfu/ml were inoculated to the broth containing 0, 0.3, 0.5 and 1% (w/v) bile salt and incubated at 37 °C under anaerobic conditions for 24 h. The growth of bacterial isolates was checked by dilution plate count on MRS agar. The survival rate of each strain was expressed as the percentage of viable cells in the presence of bile salt compared to that without bile salt. The experiment was performed in three replicates and the mean values were calculated.

3.2.5 Determination of antibacterial activity

The antibacterial activity of the selected isolates was determined by agar spot-on-lawn test introduced by Schillinger and Lücke (1989) and Mante et al (2003) with some modification. The indicator bacteria used in this study were *Escherichia coli* TISTR 780, *Salmonella typhi* DMST 5784, and *Staphylococcus aureus* TISTR 029. One µl of each overnight culture of selected lactobacillus was spotted on MRS plates (containing 0.2% glucose and 1.2% agar) and incubated under anaerobic condition for 48 h to develop colony. A portion of 0.25 ml of 1:10 dilution of an overnight culture of the indicator bacteria was inoculated in 9 ml of Brain Heart Infusion soft agar (0.7% agar). The medium was immediately poured over the MRS plate on which the tested lactobacillus was grown. The plates were incubated aerobically at 37 °C for 24

h. The antibacterial activity was determined by measuring the diameter of inhibition clear zone and growth spot with caliper. The inhibition activity of the test strains was expressed as the difference of these two diameters.

3.2.6 Deterimination of cell surface hydrophobicity

The in vitro cell surface hydrophobicity was determined by the bacterial adherence to hydrocarbon assay modified from the methods of Rosenberg et al. (1980). Briefly, the test strains were grown in MRS broth at 37 °C under anaerobic condition. The 18-24 h (stationary phase) test culture was harvested by centrifugation at 6,000 rpm for 10 min, washed twice and resuspended in 50 mM K_2HPO_4 (pH 6.5) buffer to an optical density (OD_{560}) of 0.8-1.0 (A_0) measured spectrophotometrically. A portion of 0.6 ml of n-hexadecane was added into 3 ml of bacterial suspension. The mixtures were blended using a vortex mixer for 120 s. The tubes were allowed to stand at 37 °C for 30 min to separate the two phases. The aqueous phase was carefully removed and the OD_{560} of the aqueous phase (A) was measured. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (%H) of the strain adhering to hexadecane was calculated using the equation as following.

$$\%H = \frac{(A_0 - A)}{A_0} \times 100$$

3.2.7 Effect of inulin on *Lactobacillus* growth (Saarela et al., 2003)

The growth of selected LAB in the presence of oligosaccharides was compared by measuring absorbances over time at 660 nm. The media used for

investigation was basal MRS broth (Appendix A) with various carbon sources or prebiotic sugar at a final concentration of 0.5% (w/v). For banana, powder of lyophilized banana was soaked in 50% ethanol (5% w/v) for 1 hour, then it was filtrated by Whatman No.4 paper filter. All carbon sources were filtrated through 0.45 μm membrane for sterilization. Anaerobic growth tests for individual cultures were conducted in sealed glass test tubes. Each tube was inoculated from an overnight culture The experiments was conducted under anaerobic conditions. Growth determined at stationary phase (24 h) on glucose was calculated as 100%.

$$\text{Growth relative to glucose} = \frac{\text{absorbance unit } (A_{660nm}) \text{ of glucose at 24 h X 100}}{\text{absorbance unit } (A_{660nm}) \text{ of prebiotic sugar}}$$

3.2.8 Identification of bacterial isolates by biochemical reactions.

Biochemical identifications of the bacterial isolates were based on the ability of the isolates to utilize or oxidize different carbon sources, as determined by API CH 50 (Biomerieux, France) and the Biolog AN MicroPlate (Biolog, Inc., Hayward, CA, USA) methods. Carbohydrate fermentation patterns were performed in the API CH50 using API 50 CHL medium. The Biolog system is an automated identification and classification system for microorganisms and is based on the test strains ability to oxidize 95 different carbon substrates which include amino aids, carboxylic acids and carbohydrates. The selected isolates were identified by using these methods according to the manufacturer's instructions.

3.2.9 Identification of bacterial isolates by molecular techniques

The lactobacilli demonstrated the highest resistant to acid and bile were selected. Each selected strain was cultivated at 37 °C in MRS broth incubated under anaerobic conditions. After 24 h incubation, 1.5 ml of the culture was centrifuged at 8,000 rpm for 7 min at 4 °C. The pellets were washed twice with 900 µl sterile normal saline solution. The supernatant was discarded and the pellets were washed again with 900 µl of sterile EDTA (50 mM, pH 8.0). The pellet products were stored at -20 °C. The MasterPure^{TM} gram positive DNA purification kit (Epicentre, Madison, USA) was used to obtain DNA samples according to *Listeria monocytogenes* performance in the instruction of the manufacturer. PCR procedure based on 16S rRNA gene sequences genus-specific, multiplex genus-specific and species-specific primers were used to validate. For genus-specific PCR, isolates were identified to the genus level using the primers LbLMA1-rev and R16-1 according to Dubernet et al. (2002). The reaction mixture (total volume 25 µl) contained 1 µl of each primer (10 mM), 10 x PCR buffer (Finnzymes, Espoo, Finland), dNTP-Mix (10 mM) (Roth, Karlshule, Germany), Dynazyme (2 U/µl) (Finnzymes, Espoo, Finland), and 0.5 µl of bacterial DNA. The PCR was conducted in a Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 94 °C for 4 min; 35 cycles consisting of denaturation at 94 °C for 1 min, annealing temperature at 55 °C for 1 min including time increment of 2 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The PCR products were separated by electrophoresis in 2% agarose gel. The gel was stained in ethidium bromide (5µg/ml) and visualized under UV light. Multiplex genus-specific PCR as described by Song et

al. (2000) was used to characterize *Lactobacillus* spp. in five district groups according to sizes of fragment products (300, 350, 400, 450 and 700 bp) with the group-specific primers Lac-2, Ldel-7, LU-5, LU-3, and LU-1. For species-specific, PCR with primers Lfer-3, Lfer-4, Reu-1, Reu-4, Lpla-2, and Lpla-3 were performed to identify *L. fermentum*, *L. reuteri*, and *L. plantarum*. The PCR products were analyzed by agarose gel electrophoresis and photographed under UV exposure.

16S rRNA sequencing of selected strains was also investigated to confirm the identification data. Bacterial cells grown in MRS broth were harvested in the late-exponential phase and DNA was extracted essentially by Isoplant DNA extraction kit according to manufacturing procedure. DNA concentrations were estimated by UV absorbance at 260 nm. Agarose gel was visualized with ethidium bromide to confirm the purity of genomic DNA. The 16S rRNA genes of the selected strains (corresponding to positions 27–1522 of the *Escherichia coli* 16S rRNA gene) were amplified by PCR using PCR Sprint Thermal Cycler. The six oligonucleotide primers from Operon (Germany) (M27F, 520R, M377F, 1080R, M920F and 1522R) used in the PCR amplification have been described previously (Mori et al., 1997). The PCR products were purified by Suprec^{TM}-02 filter tube (Takara Shuzo). The purified PCR products were sequenced by First Base Laboratories (Malaysia). The resulting sequences were subjected to similarity searches against sequences within the public databases, to determine a possible phylogenetic classification for the selected strains. To determine the closest known relatives of the novel strains, based on 16S rDNA sequences, primary searches were performed in GenBank using the FASTA and BLAST program (The National Centre for Biotechnology Information; NCBI; http://www.ncbi.nlm.nih.gov/).

3.2.10 Antibiotic susceptibility test

The antibiotic susceptibility test used in this study was agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) (2000). The determination of minimum inhibitory concentration (MIC) to certain antimicrobial agents recommended by Scienctific Committee on Animal Nutrition (SCAN) comprised ampicillin, chloramphenicol, ciprofloxin quinupristin, erythromycin, gentamycin, kanamycin linezolid, rifampicin, streptomycin, tetracycline, and vancomycin using Mueller-Hinton agar (Merck, Darmstadt, Germany) in anaerobic condition was carried out. Briefly, the sterilized agar was allowed to reach 50 °C in a water bath. Dilution series of antimicrobial agents were prepared in suitable solvents and diluents. Add 1 ml of working solution in 9 ml of molten agar, mix thoroughly, and poured the agar into sterile petri dishes. The agar plates were allowed to set at room temperature. Inoculum was prepared by suspending several bacterial colonies from a fresh agar plate in normal saline to a McFarland 0.5 turbidity standard (containing 1-2 x 10^8 cfu/ml). Then, the 0.5 McFarland suspensions were diluted 1:10 in normal saline to obtain a concentration of 10^7 cfu/ml. A spot of 1 μl of the inoculum was placed on the agar surface yielding approximately 10^4 cfu/spot. The inoculated plates were allowed to stand at room temperature about 30 min. The plates were moved to 37 °C incubators under anaerobic condition for 24 h. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited growth, disregarding a single colony or faint haze caused by the inoculum.

3.2.11 Haemolytic activity

For testing haemolytic activity, fresh lactobacilli cultures were streaked on Columbia agar plates, containing 5% (v/v) sheep blood, and incubated for 48 h at 30 °C. Blood agar plates were examined for signs of *β*-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or *γ*-haemolysis (no zones around colonies).

3.2.12 Safety study of viable lactobacilli

The study protocol had been approved by the Ethical Review Committee of Faculty of Medicine, Chiang Mai University. In this study, the selected isolates and *Lactobacillus delbruckii* ssp.*bulgaricus* TISTR 892 were used. The bacteria were inoculated on MRS agar and incubated at 37 ^{o}C for 48 h. A single colony was inoculated in 10 ml of MRS broth and overnight incubated at 37^{o}C. Bacterial cells were harvested by centrifugation at 10,000 rpm for 7 min and washed twice with sterile distilled water. The cells were resuspended in sterile distilled water at two concentrations (10^{7} and 10^{11} cfu/ml), based on the OD value (at 600 nm) evaluated preliminarily.

Male Swiss albino mice with the age of 5 weeks old and 30-35 g body weight obtained from the National Laboratory Animals Center, Mahidol University, Nakhonpathom province, Thailand were used in this study. All mice were acclimatized to the conditions in animal laboratory for a week prior to the experiment. Mice were housed in cages at 22 ± 2 ^{o}C under 12/12 h light/dark cycle. Food and water were provided ad libitum. The safety study used was based on the protocol of Park et al. (2005) with some modification. The animals were assigned to nine

treatment groups, each containing eight male mice. The experimental groups were administered intragastrically by a stainless steel feeding needle and 1 ml syringe with different doses (10^6 and 10^{10} cfu/0.1 ml) of the test lactobacilli suspension, once a day for 14 days whereas the control group was treated similarly but with only sterile water. The mice body weight was measured daily (Balance model: Sartorius BP160). Clinical signs, such as diarrhea, ruffled fur, and lethargy were checked twice a day. The comparisons were analyzed by the multiple range tests. Differences were judged to be statistically significant when P value < 0.05.

3.2.13 Effect of cryoprotectants

The selected isolates and *Lactobacillus delbruckii* subsp.*bulgaricus* were used in this study. The overnight MRS broth culture (early stage of stationary phase) was harvested by centrifugation at 10,000 rpm for 7 min and then washed twice with normal saline. Cell pellets were resuspended in a solution containing different kinds of cryoprotectant such as skimmed milk, skimmed milk with one of the following additives; glycerol, sucrose, sodium glutamate, glucose, and lactose to obtain a cell concentration of 10^8–10^9 cfu/ml. An aliquot of 0.3 ml of each bacterial suspension was placed into a set of sterile 1.5-ml microcentrifuge tubes, frozen for 16 h at -20 °C and dried for 24 h in a lyophilizer at an condenser temperature of -54 °C, and a pressure of 0.090 mbar. Viable cell counts were determined before and immediately after lyophilizing. Each sample of the lyophilized bacteria was rehydrated to its original volume with saline solution. Appropriate dilution of bacterial suspension was spread on MRS agar and the plates were incubated at 37 °C for 48 h. Cell survival

was calculated as the ratio between the numbers of viable cells after to before lyophilization.

3.2.14 Tableting

3.2.14.1 Bacterial strains and preparation of powder culture

LAB used in this experiment were *Lactobacillus acidophilus* La5 and *Lactobacillus fermentum* FTL 2311. Lyophilisate form of *L.acidophilus* La5 were supplied from Chr.hansen Biosystems (Horsholm, Denmark). Briefly, *L. fermentum* FTL 2311 isolated from fermented leaves of tea (*Camellia sinensis* Linn.) was used as the model probiotic bacteria in this experiment. The culture of *L. fermentum* 2311 was grown in MRS broth (Merck, Damstadt, Germany) at 37 °C under anaerobic conditions. The culture was harvested at the beginning of stationary phase and collected by centrifugation. The harvested cells were suspended in 10% skimmed milk solution. The cultures were then frozen at -20 °C for about 12 h and subsequently freeze-dried by a freeze dryer model Chirst 1-4 (Chirst; Osterode, Germany) for 24 h. The lyophilized probiotic cultures were slightly ground into fine powders and stored at 4 °C in closed containers for further experiment in tableting process. The number of bacterial cells in lyophilized probiotic powder was between 10^{10}-10^{11} CFU/g of powder.

3.2.14.2 Test of bacterial viability in tablets exposed to a test medium

- Exposure of tablets to a test medium

According to the methods described by Chan and Zhang (2005) with some modification, the test tablets were transferred into 600 ml of 0.04 N hydrochloric acid solutions (pH 1.5) or phosphate buffer solution (PBS pH 6.8: K_2HPO_4 3.4 g/l; Na_2HPO_4 3.53 g/l). USP paddle method for dissolution test was applied by using paddle speed of 100 rpm at 37 °C. After the end of incubation period, the medium was removed and the viable cells inside the non disintegrated tested tablets were determined.

- Viability assay of cells inside the tablet

According to the method of Ferreira et al. (2005) with some modification, each tablet was broken and dispersed in 600 ml of a phosphate buffer solution (PBS) pH 6.8. A serial dilution of this suspension was made until suitable cell concentration was obtained. The cell suspension was then spread onto the pre-dried MRS agar (Merck, Damstadt, Germany) plates. The plates were incubated at 37 °C for 48 h. This plating procedure was carried out in triplicates. Colonies of bacteria were counted and converted to log CFU (colony forming unit). The survival of probiotic cells reported as percentage of viability was calculated according to the following equation,

$$\text{Viability (\%)} = \frac{\text{Number of CFU after exposure to the test medium}}{\text{Number of CFU before exposure to the test medium}} \text{ X100}$$

3.2.14.3 Tablet evaluation

The probiotic tablets obtained were evaluated for their disintegration, hardness and friability according to USP. Disintegration of the tablets was examined by means of Pharma-Test dissolution apparatus. The tablets were placed separately in the test chamber, and then immersed in PBS pH 6.8 as the disintegration medium at 37 °C for 5 h. The tablet hardness was determined by using Pharma-Test PTB 311 hardness tester. The Tablet friability was measured by using a friabilitor (Pharma-test; Type PTFE).

3.2.14.4 Effect of compression force on lactobacilli powder

Lactobacillus acidophilus La5, *L. acidophilus* 72-4, and *L. fermentum* 2311 were used in this experiment. The freeze-dried cell powders with 1% lubricants (magnesium stearate and talcum) were filled into a die with a diameter of 10 mm. Compression of the powders was performed using a flat-faced punch, single punch tablet press (Korsch EKO, Berlin, Germany), under constant environmental conditions (35%R.H.; 20-22 ^{o}C). The effect of tableting pressure on survival of probiotic bacteria was studied by compressing the powders under different pressures from 0 to 20 kN. The viability of cell powders and the probiotic tablets were examined by dilution plate count technique.

3.2.14.5 Effect of excipients

1) Tablets containing hydroxypropylmethyl cellulose phthalate (HPMCP)

The different formulations of lactobacilli tablet containing excipients (HPMCP, sodium alginate, apple-pectin, and Metolose®) were prepared by direct

compression **(Table 2)**. Magnesium stearate and talcum were used as lubricants. Tablets were compressed by an instrumented single punch tablet press (Korsch EKO, Berlin, Germany), under constant environmental conditions (35%R.H.; 20-22 ^{o}C). An exactly weighed quantity of the powder mixture was filled into a die of 10 mm diameter and hydraulic pressure was applied to form the tablets. All tablets had a plane surface. Tablets were evaluated for their acid resistance, hardness and disintegration properties as described under 3.2.14.2.

Table 2 Formulations of LAB tablet containing HPMCP and sodium alginate, apple-pectin, or Metolose®

Formulation no.	Compression force (kN)	Compositions (mg)				
		LAB	HPMCP	Na alginate	Pectin	Metolose®
A1	5	25	200	-	-	-
A2	5	33	192	-	-	-
A3	5	50	175	-	-	-
A4	5	75	150	-	-	-
A5	5	100	125	-	-	-
A6	20	100	40	-	-	-
A7	20	100	55	-	-	-
A8	20	100	70	-	-	-
A9	20	100	85	-	-	-

Table 2 (continued)

Formulation no.	Compression force (kN)	Compositions (mg)				
		LAB	HPMCP	Na alginate	Pectin	Metolose®
A10	20	100	100	-	-	-
A11	20	100	125	-	-	-
A12	10	100	40	-	-	-
A13	10	100	55	-	-	-
A14	10	100	70	-	-	-
A15	10	100	85	-	-	-
A16	10	100	100	-	-	-
A17	10	100	125	-	-	-
A18	5	100	40	-	-	-

Table 2 (continued)

Formulation no.	Compression force (kN)	Compositions (mg)				
		LAB	HPMCP	Na alginate	Pectin	Metolose®
A19	5	100	55	-	-	-
A20	5	100	70	-	-	-
A21	5	100	85	-	-	-
A22	5	100	100	-	-	-
A23	2	100	40	-	-	-
A24	2	100	55	-	-	-
A25	2	100	70	-	-	-
A26	2	100	85	-	-	-
A27	2	100	100	-	-	-

Table 2 (continued)

Formulation no.	Compression force (kN)	Compositions (mg)				
		LAB	HPMCP	Na alginate	Pectin	Metolose®
A28	2	100	125	-	-	-
A29	5	100	24	16	-	-
A30	5	100	33	22	-	-
A31	5	100	42	28	-	-
A32	2	100	24	16	-	-
A33	2	100	33	22	-	-
A34	2	100	42	28	-	-
A35	5	100	24	-	16	-
A36	5	100	33	-	22	-

Table 2 (continued)

Formulation no.	Compression force (kN)	Compositions (mg)				
		LAB	HPMCP	Na alginate	Pectin	Metolose®
A37	5	100	42	-	28	-
A38	2	100	24	-	16	-
A39	2	100	33	-	22	-
A40	2	100	42	-	28	-
A41	5	100	24	-	-	16
A42	5	100	33	-	-	22
A43	5	100	42	-	-	28
A44	2	100	24	-	-	16
A45	2	100	33	-	-	22
A46	2	100	33	-	-	28

2) Tablets containing inulin and banana powder

LAB used in this work were *Lactobacillus acidophilus* 72-4 and *Lactobacillus fermentum* 2311. Lyophilisate form of *L.acidophilus* 72-4 was supplied from Chr.hansen Biosystems (Horsholm, Denmark). Raftiline® (Orafti Active Food Ingredients, Oreye, Belgium), a commercial food grade of inulin was used in this study. Two kinds of bananas namely, Klouy Hom (Gros Michael or *Musa* AAA group) and Klouy Nam Wa (pisand Awak or *Musa* ABB group) were carried out. Edible portion of ripe banana was cut and subsequently lyophilized. The lyophilized banana was following ground to fine powder. The formulations of LAB with/without the prebiotics and banana powders (as shown in **Table 2**.) were compressed into tablets with the compression force of 8 kN. Magnesium stearate and talcum were used as lubricants. The tablet making and the evaluation of tablet properties were the same as mentioned under 3.2.14.2.

Table 3 Formulations of tablet containing inulin and banana powders

Formulation	Amount (%)						
	LAB	Inulin	lactose	Kluai Hom	Kluai NamWa	Avicel	Tablet wt. (mg)
I	10	89	-	-	-	-	385
B1	10	-	-	89	-	-	385
B2	10	-	-	89	-	-	225
B3	10	-	-	89	-	-	300
B4	10	-	-	70	-	19	225
B5	10	-	-	70	-	19	300
B6	10	-	-	70	-	19	385
B7	10	-	10	79	-	-	225
B8	10	-	10	79	-	-	300
B9	10	-	10	79	-	-	385
N1	10	-	-	-	89	-	385
N2	10	-	-	-	89	-	225
N3	10	-	-	-	89	-	300
N4	10	-	-	-	70	19	225
N5	10	-	-	-	70	19	300
N6	10	-	-	-	70	19	385
N7	10	-	10	-	79	-	225
N8	10	-	10	-	79	-	300
N9	10	-	10	-	79	-	385

B : Klouy Hom (Gros Michael or *Musa* AAA group)

N : Klouy Nam Wa (Pisang Awak or *Musa* ABB group)

3) Tablets containing hydroxypropylmethyl cellulose phthalate (HPMCP) and banana powder

L.fermentum 2311, isolated from fermented food, was used in this study. The 2311 strain was lyophilized by a lyophilizer model Chirst 1-4 (Chirst, Osterode, Germany). The tablet formulations were shown in **Table 3.** The formulation powder mixtures were compressed into tablets with the compression force of 5 kN. The tablet making and the evaluation of tablet properties were the same as mentioned previously.

3.2.14.6 Stability test of probiotic tablets

The best probiotic tablet formulation was selected to investigate in this study. The tablets were kept in tight light resistant container at 10 °C and 30 °C for 6 months. The stability of bacterial cells in terms of cell viability in the tablet along the storage period of time was investigated monthly. The method used to determine cell viability was plating procedure.

Table 4 Formulations of tablet containing HPMCP and banana powder

compositions	Content (%) in formulations																	
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
Bacteria	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
HPMCP55	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
$CaHPO_4$	74	74	54	54	64	64	-	-	-	-	-	-	-	-	-	-	-	-
Avicel	-	-	20	20	-	-	-	-	20	20	-	-	-	-	20	20	-	-
Na alginate	-	-	-	-	10	10	-	-	-	-	10	10	-	-	-	-	10	10
Lactose	-	-	-	-	-	-	74	74	54	54	64	64	-	-	-	-	-	-
Banana powder	-	-	-	-	-	-	-	-	-	-	-	-	74	74	54	54	64	64
Tablet weight (mg)	225	300	225	300	225	300	225	300	225	300	225	300	225	300	225	300	225	300