CHAPTER 3

MATERIALS AND METHODS

Study of lactobacilli strains

3.1 Fermented foods collection and LAB isolation

Thai fermented food products containing vegetable, fruit, soybean, fish or pork, fermented plant beverage and pickle were collected from north and northeast regions of Thailand as sources of lactobacilli. Total 480 fermented samples were selected over 22 provinces included the north areas such as Chiangmai, Chiangrai, Lumphun, Lumpang, Maehongson, Nan and Tak , and the northeast areas such as Burirum, Kalasin, Khonkaen, Loei, Mahasarakham, Mukdahan, Nakhonphanom, Nakhonratchasima, Nongkhai, Roi et, Sakonnakorn, Srisaket, Surin, Ubonratchathani and Udonthani.

Each sample was mixed well and randomly separated 25 g or 25 ml diluted into 225 g or 225 ml Phosphate buffer saline (PBS, pH 7.2±0.2). The amount 1 ml of appropriate decimal dilution was poured into 25 ml molten (45-50°C) de Man Rogosa Sharpe (MRS) agar (Difco Detroit, USA) with modified by containing 0.05 % (w/v) bromocresol purple (Biochemika, Fluka, Germany). Then, the solidify MRS agar plates were incubated anaerobically at 37°C for 24-48 h. A number of lactic acid bacterial strains (yellowish colony) were isolated, purified and primarily

identified for lactobacilli by phenotypic criteria such as colony morphology, Gram's stain, catalase test and carbohydrate fermentation (Kandler and Weiss, 1986). Each working purified lactobacilli strain was transferred to MRS slant tubes and it was stored at 4°C and sub-cultured every 2 weeks. Stock cultures were maintained in 40% (v/v) glycerol at –70°C. The organisms were activated 3 times in MRS broth (Difco Detroit, USA) using 1% (v/v) inoculum at 37°C for 24 h before experimental use.

3.2 Study for key functional properties and some assuring safety of isolated LAB strains

3.2.1 Bile salt and various pH tolerance

The bile salt tolerance test was modified from Du Toit et al. (1998) and Brink et al. (2006). The acid tolerance of each strain was measured using a modified method of Brink et al. (2006). Bile salt tolerance tests, MRS agar plates with and without bile salt were prepared. The pH of the bile salt agar plates were adjusted to 6.5. Overnight culture of each strain was streaked on MRS agar with 0.15% and 0.30% (w/v) bile salt (Sigma, USA). In case of pH tolerance, overnight cultures were inoculated into MRS broth with pH previously adjusted to 2, 3, 4, 5, 8 and 9 with HCl or NaOH. At the time of 0 and 2 h incubation, each strain was cultured into molten (45-50°C) modified MRS agar. The culture plates of bile salt agar and MRS gar were incubated anaerobically at 37°C for 24-72 h. The growth strain was mentioned as bile salt tolerant and pH tolerant strains, respectively. The results of

pH tolerance were shown as the percentage of viable strains in MRS agar compared to the initial plate count at 0 h. All tests were assayed in triplicate.

3.2.2 Starch, protein and lipid utilization

The strains with acid and bile salt condition survival were selected for this assay. The evaluation of activities of starch, protein and lipid utilization of lactobacilli strains, was slightly modified from methods of Tamang and Sakar (1996), Santivarangkna (1999) and Essid et al. (2009). The starch, protein and lipid utilization were assessed using agar plates supplemented with 1% (w/v) soluble starch, 10% (w/v) skim milk and 1% (v/v) tributyrin, respectively. Overnight culture of bacterial strains in MRS broths were collected by centrifugation and washed twice with PBS (pH 7.2). Then, cell pellets were resuspended and spotted onto each agar medium and incubated anaerobically at 37°C for 48-72 h. The activity of starch, protein and lipid utilization was investigated by the clear zone surrounding bacterial culture on MRS agar supplemented with starch or skim milk or tributyrin.

3.2.3 Antimicrobial activity to tested microorganisms

Antagonistic activities of lactobacilli on microbial indicators were adapted from the method of Chin et al. (2001) and Mante et al. (2003). The selected strains with bile and acid-base tolerance were cultured overnight before assay. The antagonistic activities of *Lactobacillus* cultures against seven microbial indicators (*Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*,

Shigella sonnei and *Candida albicans* ATCC 90028, which were provided by the Department of Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University were prepared into three fractions; normal cell supernatant, cell supernatant pH 7.0 and cell supernatant pH 7.0 containing 1 mg/ml catalase (Sigma, USA). Lactobacilli strains were investigated for antimicrobial activities by using the agar well diffusion technique. Bacterial tests were grown in Mueller Hinton broth (MHB) (Becton Dickinson, USA) for 24 h at 37^{o}C. In case of yeast, *C. albicans* was cultured in Sabouraud Dextrose broth (SDB) (Merck, Darmstadt Germany) for 24 h at 37^{o}C. Before the antimicrobial assay, the pathogens were prepared at equivalent to optical density 0.5 at 600 nm (OD_{600}) and diluted with phosphate buffer pH 7.2. The pathogenic bacteria and yeast with concentration approximately 10^6 and 10^5 cfu/ml, respectively, were used for the antagonistic activity assay. 100 µl sterilized filtrate supernatant was filled into the well against target microorganisms. After 24-48 h of incubation time, the diameter of the inhibition zone was measured and scored. The representation of inhibition zone were not included 7 mm diameter of well. The inhibition zone was scored as follows: larger than 6 mm equals strong inhibition (+++), between 3 and 6 mm equals moderate inhibition (++), and less than 3 mm equals weak inhibition (+).

3.2.4 Sensitivity to antibiotics of selected lactobacilli strains

The selected lactobacilli strains with previous results were further studied for their susceptibilities to antibiotics. The antibiotic susceptibility of lactobacilli strains was determined towards the following 9 antibiotics in common use included ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin,

streptomycin, tetracycline and vancomycin (all antibiotics used were purchased from Oxoid). The sensitivity to antibiotics was determined by measuring minimum inhibitory concentrations (MICs) using a microdilution titer plate according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS), which is now known as the Clinical and Laboratory Standards Institute (CLSI). The serial dilutions of antibiotics were prepared with the concentration from 0.06 to 256 µg/ml in warm molten MRS agar. The strains were grown in MRS broth at 37ºC for 18-24 h, after which they were adjusted for turbidity to McFarland No. 0.5. The amount of 1 µl bacterial suspension at approximately concentration of $1x10^8$cfu/ml was inoculated on each agar plate containing diluted antibiotics. Incubation was conducted at 37^{o}C for 24 h in an aerobic condition. The MIC was evaluated as the lowest concentration of the antibiotic agent that completely inhibited growth of bacterial strains, showing a limpid area. The breakpoints in this study were defined as the MICs values recommended by the Scientific Committee on Animal Nutrition (SCAN) (EC, 2001). Strains with MICs values less than the breakpoints are considered as susceptibility. On the contrary, the values equal to or higher than the breakpoints are considered as resistance.

3.2.5 Haemolytic activity of lactobacilli strains

This method was slightly modified from the method of Uymaz et al. (2009). Each activated culture of lactobacilli was streaked on tryptic soy agar (TSA; Merck, Germany) plates supplemented with 5% (v/v) human blood, and was then incubated anaerobically at 37°C for 24-72 h. Blood agar plates were investigated for

result of β-haemolysis (halo zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies) activity.

3.2.6 Bile salt hydrolase (BSH) activity

The BSH activity determination was applied from Du Toit et al. (1998) and Lim et al. (2004). The growth into stationary phase of each isolate was investigated for the bile salt hydrolase activity by streaking on MRS agar plate supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma, USA) and 0.37 g/l of $CaCl_2$ (Merck, Germany). The inoculated plates were incubated anaerobically at 37°C for 72 h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of lactobacilli.

3.2.7 *In vitro* cholesterol-lowering property of active cell-free broth

The selected strains from previous assays were investigated for cholesterol-lowering activity. Growth cultures of strain (1%) were inoculated into freshly prepared MRS supplemented with 0.3% oxgall (w/v) (Sigma, USA) as a source of bile salt. Standard cholesterol in water-soluble form (polyoxyethanyl cholesteryl sebacate; Sigma, USA) was sterilized, filtered and added to MRS broth at a final concentration of 70 to 100 μg/ml. After 24 h of growth, the final pH of active cell culture was measured. Then, the cells were centrifuged. Spent broth was then sterilized by using a 0.45 μm membrane filter (Pall Gelman Laboratory) as cell-free broth of active cells. The total amount of cholesterol in both the active cell-free broth and cell pellet were determined. An un-inoculated MRS broth at the same

condition was a negative control. The cholesterol levels were determined by a small modification of the method of Rudel and Morris (1973) and Gilliland et al. (1985). The activity of cholesterol-lowering was calculated as a percentage by the treatment compared with the control (MRS broth supplemented 0.3% oxgall) as follows: [1-(residual cholesterol in cell-free broth)/(cholesterol of control broth)]×100.

3.2.8 *In vitro* cholesterol-lowering property of inactive (dead and resting) cell free broth

The method used was modified from Kimoto et al. (2002), Liong and Shah (2005a) and Marculescu et al. (2005). After 24 h of cell growth in MRS broth supplemented with 0.3% oxgall, cells were harvested by centrifuging at 10,000 × *g* at 4°C for 10 min. The cell pellets were washed twice with sterile distilled water and prepared into two fractions. For the resting cells preparation, the cell pellets were suspended with phosphate buffer saline (pH 7.0±0.2) containing 0.3% oxgall and standard water-soluble cholesterol. For the second fraction, cell pellets were suspended with sterile distilled water and autoclaved at 121°C for 15 min. After being autoclaved, the cell pellets were suspended with MRS broth containing 0.3% oxgall and standard water-soluble cholesterol for dead cell preparation. The dead and resting cells were incubated at 37°C for 24 h. Spent broths and cell pellets of these fractions were assayed for cholesterol-lowering content as previously described.

3.2.9 Assay for growth curve and *β*-glucosidase activity

The lactobacilli strains with previous tests were continually investigated for growth curve and *β*-glucosidase enzyme activity. Each lactobacilli strain was activated and was then inoculated with 3% (v/v) into 10 ml MRS broth for individual time point in glass-covered tube. The lactobacilli inoculum tubes were incubated anaerobically at 37°C and each tube per time point (h0, h12, h18, h24, h36 and h48) was taken to determine the growth curve. Growth curve of each lactobacilli strain was measured the optical density at 620 nm using Spectronic Genesys2 (Milton Roy, USA). For assays, the enzyme activities were performed with two fractions included cell-free and cell associated *β*-glucosidase activities. Lactobacilli strains were grown in MRS broth for 12, 18, 24, 36 and 48 h at 37°C. Supernatants and cells of bacterial culture were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. The cell pellets were washed twice and resuspended with 0.1 M sodium phosphate buffer (pH 7.0). The β-glucosidase activity was determined by measuring the rate of hydrolysis of ρ-nitrophenyl-β-D-glucopyranoside (ρNPG, Sigma Chemical Co., Mo., USA) slightly modified from the method described by Otieno et al. (2006a). The concentration of 1 mM ρNPG dissolved in 0.1 M sodium phosphate buffer (pH 7.0) was added to cell-free supernatant and cell suspension, then the mixture were incubated for 30 min at 37°C. The reactions were stopped by adding 0.5 M of cold sodium carbonate (4°C). The supernatants of reaction were obtained by centrifugation at 5,000 × *g* for 10 min at 4°C. The supernatants were immediately measured at 405 nm using multimode spectrophotometer (DTX 880, Beckman Coulter Inc., UK). The amount of ρ-nitrophenol released in the supernatants was evaluated. One unit of the extracellular enzyme activity was defined as the amount of β-glucosidase that released 1 μmol of ρ-nitrophenol from the substrate ρNPG per minute under the condition of analysis.

3.2.10 *In vitro* adherence inhibition assay

The one selected lactobacilli strain was investigated for the antagonistic activity. A monolayer of Caco-2 cells (originating from human colorectal carcinoma) was used to evaluate the antagonistic activity of lactobacilli strain against three bacterial tests. The enteropathogenic bacteria such as *Salmonella typhi* and *Shigella sonnei* and representative intestinal bacteria as *E. coli* ATCC 25922 were investigated as bacterial tests (three bacteria were provided by the Department of Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University). The Caco-2 cells were obtained from the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand. The Caco-2 cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco^{TM}, Invitrogen corporation, USA) supplemented with 10% heat-inactivated (30 min, 55^{o}C) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37^{o}C in an atmosphere of 5% CO_2 and 95% relative humidity. The Caco-2 cells with a final concentration of $2x10^5$cells/well in 24-well SPL cell culture plates (SPL Life Sciences, Korea) were used. For the adherence inhibition assay, the method was slightly modified from Forestier et al. (2001), Lee et al. (2003) and Gueimonde et al. (2006). The adherence inhibitions of lactobacilli strain against pathogens were evaluated with three ways as elimination, competition and displacement. For the elimination assay, the monolayer of Caco-2 cells was incubated with $1.2x10^8$ cfu/well of lactobacilli strain for 1 h (lactobacilli pre-inoculation) in RPMI medium 1640 (Gibco^{TM}, Invitrogen corporation, USA). After washing three times with PBS, 10^7 cfu/well of each pathogen was inoculated into Caco-2 cells and continually incubated for 1 h in RPMI medium 1640. For

competition assay, the amount 1.2x 10^8 cfu lactobacilli strain per well and 10^7 cfu/well of each pathogen were mixed and inoculated to monolayer Caco-2 cells (co-culture of lactobacilli and the pathogen). Then, the co-culture was incubated for 1 h in RPMI medium 1640. For the displacement assay, firstly the monolayer Caco-2 cells and each pathogen were co-cultured for 1 h. After washing three times with PBS, the lactobacilli was inoculated into Caco-2 cells and incubated for further 1 h (*Lactobacillus* post-inoculation). For all three assays, the co-culture between Caco-2 cells and bacteria were washed triplicate with PBS and lysed with 0.05% (v/v) Triton X-100 (Sigma-Aldrich, USA). The inhibitions of three bacterial tests adhesion were evaluated with the number of lactobacilli strain cultured on MRS agar, *S. typhi* and *S. sonnei* on *Salmonella-Shigella* agar and *E. coli* on Eosin Methylene blue agar by the serial dilutions plating method. The results were demonstrated as the percentage of treated bacterial test adherence compared to the adhesion of only bacterial test (100% adherence). The data points are displayed as means ± standard deviation (SD).

3.2.11 Dead cell lactobacilli affected adhesion on Caco-2 cells and induced interleukin-6, interleukin-10 and interleukin-12 production

The effects of heat-killed lactobacilli of the one selected strain on adhesion to Caco-2 and the immune response via observation of IL-6, IL-10 and IL-12 were determined. *L. acidophilus* TISTR 450 and *L. casei* subsp. *rhamnosus* TISTR 047 were obtained from Thailand Institute of Scientific and Technological Research (TISTR). *Lactobacillus* sp. (LCC1) and *Lactobacillus* sp. (LCC2) were obtained

from Health Product Research Unit, Chiang Mai University. Four bacterial strains were determined as control. Lactobacilli strains were cultured in MRS broth at 37°C. Besides, *E. coli* ATCC 25922 was cultured in Tryptic soy broth (TSB; Merck, Germany) at 37°C. The growth profile of each microorganism was investigated and expressed as the value of specific growth rate (μ). Optical density (OD_{600}= 0.5) of bacterial suspension was calculated; the bacterial cell number from the growth curve (data not shown) gave approximately 10^8 cfu/ml. Bacterial cells were harvested and washed three times with PBS solution, and suspended in distilled water. These bacterial cells were heated at 75°C for 30 minutes to obtain heat-killed *L. acidophilus* TISTR 450 (HK-LA450), heat-killed *L. casei* subsp. *rhamnosus* TISTR 047 (HK-LC047), heat-killed *Lactobacillus* sp., LCC1 (HK-LCC1) heat-killed *Lactobacillus* sp., LCC2 (HK-LCC2), heat-killed selected lactobacilli strain and heat-killed *Escherichia coli* ATCC 25922 (HK-EC). The Caco-2 cells were routinely grown in the medium same described in section 3.2.10.

For adhesion assay, Caco-2 monolayers which were modified from Murosaki et al. (1998), Tuomola and Salminen (1998) and Morita et al. (2002), were prepared in 25 cm^2 tissue culture flasks. Cells were subcultured every 2 days. Monolayer cells were seeded up to 80% confluence, gram stained with tryphan blue and counted. Adjusted cell to final concentration 10^4 cells/ml to obtain confluence used in adhesion assays. The adherence of heat-killed bacterial strains to Caco-2 cell cultures was examined by adding HK-LA450, HK-LC047, HK-LCC1, HK-LCC2, HK selected lactobacilli and HK-EC into well with concentration 10^8 cfu/ml. After incubation, the monolayers were washed three times with sterile Triton X-100, fixed with methanol, gram stained, and examined microscopically. The numbers of heat-killed bacteria attached to Caco-2 cells was counted. Each sample was conducted in

triplicate and each time was assessed in 20 randomly chosen microscopic fields. The adhesion ratio (%) was calculated as follows:

$$\%Adhesion = \left[\frac{averageh2}{averageh0}\right] x100$$

Where, average h2 and average h0 were the amount of the cell at 2 h and initial time, respectively.

Cytokine-induction of heat-killed bacteria assay was modified from Murosaki et al. (1998), Morita et al. (2002) and Sashihara et al. (2006). Briefly, Caco-2 cells were seeded on sterile 24 well flat-bottom plates at a concentration of 10^4 cells/ml. Then, all heat-killed tested strains (10^8 cfu/ml) were added and incubated at 37°C in an atmosphere of 5% CO_2 95% air. After 24 h incubation, the cell culture supernatants were centrifuged in order to remove cells. To analyze cytokine induction, supernatants from treated bacterial cells were collected. The concentration of the cytokines IL-6, IL-10 and IL-12 in the supernatant were determined using commercially available enzyme linked immunosorbent assay (ELISA) kit (Quantikine® IL-6, 10 and 12 Immunoassay, USA).

3.3 Identification of selected lactobailli strain with functional properties

Lactic acid bacteria (LAB) which was selected strain in this experiment was characterized the morphology by scanning electron microscopic method and the carbohydrate fermentation profile using API 50 CHL strip tests (Biomerieux, France) to characterize carbohydrate fermentation profiles. Then, the selected

strains were identified according to the method of Byun et al. (2004), Massi et al. (2004) and Tamminen et al. (2004). Lactobacilli strains were inoculated in MRS broth overnight at 37^{o}C. The cell pellet was harvested by centrifuging and washed twice with PBS. The nucleic acid was extracted from the cell pellet using the UltraClean^{TM} soil DNA isolation kit (Mo Bio Laboratories, Inc, Canada) and also purified using the high pure PCR template preparation kit (Roche, USA). PCR procedures based on 16S ribosomal DNA sequences lactobacilli genus-specific and lactobacilli species-specific primers were used for identification. The PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The primers used for the amplification of the 16S rDNA were 5'-GCCGCCTAAGGTGGGACAGAT-3' (forward primer) and 5'-TTACCTAACGGTAAATGCGA-3' (reverse primer) (Massi et al., 2004). Amplification was done by initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec. The annealing temperature of the primers was 60°C for 30 sec and extension at 72°C for 30 sec. Final extension was at 72°C for 5 min. For the sequencing, the strain was sent to the KU-Vector Custom DNA Synthesis Service, Kasetsart University, Bangkok, Thailand. The partial 16S ribosomal DNA sequence of lactobacilli strain was sequenced using the BigDye Terminator Cycle Sequencing Kit and sequencing machine model ABI 377 (PE Applied Biosystem). The homology search of the 16S rDNA was blasted in the National Centre for Biotechnology Information (NCBI) GenBank with the BLASTN program (http://blast.ncbi.nlm.nih.gov/blast.cgi).

Study of fermented soybean milk by using selected lactobacilli strain as functional starter

3.4 Soybean milk preparation and fermentation (modified from Bordignon et al., 2004; Wang et al., 2006)

Soybean CM 60 is popular variety in northern Thailand due to it can adapt to wide range of environment and provides high yield. The CM 60 can be cultivated both in dry and raining seasons. Furthermore, the proximate compositions and isoflavone contents were detected largely levels. Therefore, the soybean CM 60 variety was selected to produce fermented soybean milk in this study. Whole soybean CM 60 variety seeds, which were obtained from Limsakdakun Co., Ltd., Chiang Mai, Thailand, were washed and then soaked in sterilized distilled water with a ratio of 1:3 at room temperature for 8 h (Sankonkit, 2007). After soaking, the soybeans were autoclaved at 121ºC for 15 min. The steamed soybeans were ground in pasteurized electric juice blender for 3 min and were then extracted with sterilized distilled water using a weight ratio at 1:8. The blended solution of soybeans was filtered through triple-layered cheesecloth. A filtrated solution was recognized as soybean milk. The obtained soybean milk was supplemented with 6% (w/v) of sucrose and was then poured into 150 ml sterile covered glass bottle (for analysis which one bottle was represented to one replication individual fermentation time) and into 500 ml sterile covered glass bottle for chemical analysis, and was then pasteurized at 69±3ºC for 20 min (Marshall and Arbuckle, 1996). After cooling, the soybean milk was prepared to inoculate with active starter culture of one selected functional lactobacilli strain. The selected one of lactobacilli strain with functional

properties was prepared as starter culture for soymilk fermentation. The activated lactobacilli culture in MRS broth was harvested for the cells by centrifuging at 10,000 rpm, 4°C for 10 min. The cell pellet was washed twice with PBS (pH 7.2±0.2). The harvested lactobacilli culture was inoculated to soybean milk with the concentration of $1x10^8$cfu/ml soybean milk. The homogeneous soybean milk with functional starter culture was incubated at 37ºC for 21 days.

3.4.1 Physicochemical analysis of soybean milk

During fermentation, samples on 0 h, 24 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of incubation were taken and analyzed for percentages of ash, moisture, protein, fat, carbohydrate, and energy (kcal) by Institute of Product Quality and Standardization (IQS), Maejo University, Chiang Mai, Thailand.

3.4.2 Growth curve of starter lactobacilli (Sirilun, 2005)

This study was carried out to evaluate the growth profiles of lactobacilli starter during fermentation of soybean milk. The soybean milk samples were taken at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation and enumerated of lactobacilli strain by pour plating the initial suspensions non-dilution and their 10-fold serial dilutions (1 ml) into the modified molten MRS agar (45-50°C). The culture plates were cultivated anaerobically at 37°C for 24-72 h. All assays were performed in duplicate plates and the data were averaged. The yellowish colonies were counted as cfu/ml soybean milk.

3.4.3 Study the change of pH and titratable acidity (AOAC, 2000)

The decrement in pH of the fermented soybean milk (at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation period) was investigated with a Consort C830 pH meter (Consort, USA). The acid titration was conducted with 0.1 N NaOH on the soybean milk (0.5 ml) using phenolphthalein (Merck, Germany) as an indicator. The results were calculated as % lactic acid (w/v) of fermented soybean milk. All data were performed in triplicate and the values were averaged.

3.4.4 Study β-glucosidase activity in fermented soybean milk

The 1 ml aliquot samples of soybean milk were taken. The *β*-glucosidase enzyme activity was determined immediately in soybean milk during fermentation at 37ºC at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation as methods described in the section 3.2.9. The 2 ml of 1 mM ρNPG dissolved in 0.1 M sodium phosphate buffer (pH 7.0) was added to 1 ml soybean milk aliquot, then the mixture were incubated at 37^{o}C for 30 min. The reactions were stopped by adding 4 ml of 0.5 M of cold sodium carbonate (4^{o}C). The supernatants of reaction were obtained by centrifugation at 15,000 rpm for 15 min at 4^{o}C. The supernatants were immediately measured at 405 nm using a multimode spectrophotometer. The amount of ρ-nitrophenol released in the supernatants was determined.

3.4.5 Study of the isoflavone content in fermented soybean milk

The soybean milk (at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation of fermentation) were extracted and determined for the contents of isoflavone according to the methods adapted from Pyo et al. (2005). Briefly, 2 ml of soybean milk samples were mixed with 8 ml of 80% aqueous methanol and sonicated at room temperature for 30 min. Ten ml of the mixture was then centrifuged at 5,000 rpm for 10 min, and clear supernatant was filtered through a 0.45 µm syringe filter membrane (Millipore, Bedford, MA, USA) prior to inject onto high performance liquid chromatography (HPLC). Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis was performed by using Hewlett Packard HP 1100 series, DAD detector, HP ChemStation software (Scientific Equipment Source, Pickering, Canada) and BSD Hypersil C18 column (250mm x 4.6mm, 5µm). The mobile phase was composed of 0.2% formic acid in filtered Milli Q water (solvent A) and 0.1% acetic in acetonitrile (solvent B). HPLC grade formic acid, acetonitrile and methanol were purchased from Sigma-Aldrich (Sigma-aldrich, MO, USA). The soybean milk samples were injected as 20 µl. The solvents flow rate was 1.0 ml/min, using gradient of mobile phase started at 90% A (10% B) at 0 min over 30 min, decreasing to 10% A for 5 min and finally increased to 90% A over 5 min until completing the gradient program of 40 min prior to next injection. Soybean milk isoflavones were detected at 254 nm and peak area responses were integrated compared to area of standard isoflavones at the same retention time. The standard isoflavone glucosides (daidzin and genistin) and isoflavone aglycones (daidzein and genistein) were purchased from Sigma-Aldrich.

3.5 Study the optimum levels of the fermented soybean milk factors to find the optimum formula that could support the probiotic culture growth profile and biological activity in fermented soybean milk at day 3 of fermentation

In this study, soybean milk samples from the preparation in the section 3.4 were studied. After the steamed soybeans were ground in pasteurized electric juice blender, the soybean milk were fermented by variation of three levels of three factors in fermented soybean milk included (1) soybean extraction with sterilized distilled water using a ratio their weight 1:5, 1:8 and 1:11, (2) amount of lactobacilli starter approximately at 10^6 cfu/ml, 10^8 cfu/ml and 10^{10} cfu/ml, and (3) amount of sucrose added with the concentration 2%, 6% and 10% (w/v) of soy milk. Three factors of soybean milk fermentation were determined by applying a 2^3 factorial experiment in central composite design (Tables 3.1 and 3.2).

Table 3.1 The levels for the three factors of soybean milk fermentation with lactobacilli starter in the 2^3 factorial experiments in central composite design

Factors	**Low level (-)**	**Center point (Cp; 0)**	**High level (+)**	**Unit**
Ratio of water per soybean in milk extraction	5	8	11	Per 1 unit of soybean (w/w)
Lactobacilli starter number	6	8	10	log cfu/ml
Concentration of sucrose	2	6	10	% (w/v)

Table 3.2 The experimental arrangement of the study of level for the three factors of soybean milk fermentation with lactobacilli starter in the 2^3 factorial experiments in central composite design

Experimental treatment	Code	Factor[a]		
		Water extraction ratios per 1 part of soybean (w/w)	Initial inoculum of LCC 150 starter (log cfu/ml)	Sucrose added to soybean milk (%w/v)
1	1	-1(5)	-1(6)	-1(2)
2	a	1(11)	-1(6)	-1(2)
3	b	-1(5)	1(10)	-1(2)
4	c	-1(5)	-1(6)	1(10)
5	ab	1(11)	1(10)	-1(2)
6	ac	1(11)	-1(6)	1(10)
7	bc	-1(5)	1(10)	1(10)
8	abc	1(11)	1(10)	1(10)
9	cp1	0(8)	0(8)	0(6)
10	cp2	0(8)	0(8)	0(6)
11	cp3	0(8)	0(8)	0(6)

[a]Values in parentheses are decoded variables

Soybean milk treatments in three center points (cp1-cp3) were prepared same as the previous study in the section 3.4

3.5.1 Study the physicochemical composition of fermented soybean milk, growth profile of starter lactobacilli, the β-glucosidase activity and isoflavone content in fermented soybean milk

The analytical methods of these parameters were processed as previous described in this study. The fermented soybean milk treatments which were simulated along the experimental designs above were studied.

3.5.2 Sensory evaluation

The selected preparation from the results of section 3.5.1 was then evaluated by 20 volunteers. The final sensory evaluation was done by 9-point Hedonic scale. The scales were as follows: dislike extremely = 1, dislike very much = 2, dislike moderately = 3, dislike slightly = 4, neither like nor dislike = 5, like slightly = 6, like moderately = 7, like very much = 8 and like extremely = 9. The final value was used for statistical calculation by ANOVA test. The score from this Hedonic scale evaluation was statistically calculated by ANOVA test.

3.5.3 In *vivo* cholesterol profile changes of lactobacilli strain and soybean milk (modified from experiment of Xiao and Kondo, 2003; Greany and Nettleton, 2004)

The one selected formula of fermented soybean milk (from section 3.5) was studied for the effect on serum cholesterol change in animal. Four-week-old male Spraque-Dawley rats were purchased from National Laboratory Animal Centre,

Mahidol University (Salaya, Nakhon Pathom, Thailand). The study was carried out under the ethical approval of the Research Ethics Committee of Faculty of Medicine (No. 20/2552 was approved on June 23rd, 2009), Chiang Mai University. The rats weight about 150-200 g were kept to acclimatize with the experimental room in a temperature controlled room (25±2ºC) for 1 week with a 12 h-light/12-h dark cycle and given *ad libitum* of drinking water and commercially prepared pellet diet. The animals were arranged into 6 groups (*n*=10) (see below) and were kept in stainless steel cage. The experimental was done for 12 weeks. The general health and body weights of rat were investigated. At the end of experimental period, the rats were anesthetized with Zoletil 50 (Virbac, France). Blood were then collected by cardiac puncture in a 6 ml-heparinated sterile tube (Vacuette, France) and immediately monitored of serum cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride (monitored by MTLab Chiang Mai, Chiang Mai). Values are expressed as the mean±SD. Statistical differences were considered significant at $P<0.05$ using ANOVA with DMRT posthoc.

Group 1: normal rats were given normal rat diet

Group 2: normal rats with daily tube feeding administration with appropriate number of selected functional lactobacilli strain (resuspended cells in sterile 0.85% (w/v) normal saline)

Group 3: normal rats with daily tube feeding administration with fermented soymilk containing lactobacilli strain

The high cholesterol rats, which were processed on group 4-6, were managed by given cholesterol-enriched diet (normal diet supplemented with 1.0% (w/w)

cholesterol (Carlo Erba) in cooked oil). Blood from rat tail was monitored cholesterol level prior experimental used.

Group 4: high cholesterol rats were given normal rat diet

Group 5: high cholesterol rats with daily tube feeding administration with appropriate number of selected functional lactobacilli strain (resuspended cells in sterile 0.85% (w/v) normal saline)

Group 6: high cholesterol rats with daily tube feeding administration with fermented soymilk containing lactobacilli strain

Statistical analysis

Collected data from the experiments were statistically analyzed by Analysis of Variance using a randomized completely block design with three replications. For the experimental design in the section 3.5, the data was statistically analyzed by Analysis of Variance (ANOVA) using a Factorial Experiment in central composite design with three replications. To determine differences between treatment means, a Least Significant Difference (LSD) test and Duncan's Multiple Range test (DMRT) were employed. All of the statistical analysis was conducted by the SPSS statistical software version 11.0 for Windows (SPSS Inc, Chicago, IL, USA). The level of significant difference was defined at $P<0.05$.