Chapter 3

Materials and Methods

3.1 Raw material

3.1.1 Black bean, Raitip brand from Tesco Lotus, Chiang Mai

3.1.2 Mung bean, Raitip brand from Tesco Lotus, Chiang Mai

3.1.3 Red kidney bean, Raitip from Tesco Lotus, Chiang Mai

3.1.4 Soy bean, Raitip from Tesco Lotus, Chiang Mai

3.1.5 Sodium Caseinate (BBA, France)

3.2 Probiotic microorganism

Lactobacillus acidophilus TISTR 450 from Thailand Institute of Scientific and Technological Research that was donated by a Japanese researcher.

3.3 Equipment

3.3.1 An autoclave (Gallenkamp, England)

3.3.2 Autopipettes (Gilson, France)

3.3.3 15 and 50 ml sterile centrifuge tubes (Corning®, USA)

3.3.4 A 25 ml titration burette (HBG, Germany)

3.3.5 Filtered cloths

3.3.6 1000 ml measuring cylinders

3.3.7 100 ml measuring cylinders

3.3.8 125 ml erlenmeyer flasks

3.3.9 250 ml erlenmeyer flasks

3.3.10 A gas cooker (National, Thailand)

3.3.11 A big spoon (Seagull, Thailand)

3.3.12 Kitchen pots (Crocodile, Thailand)

3.3.13 0-100^{O}C mercury thermometer (Fisher, UK)

3.3.14 An oven (Gallenkamp, England)

3.3.15 A pH meter (Consort, Belgium)

3.3.16 Measurement pipettes (10 ml) (HBG, Germany)

3.3.17 A hand refractometer (ATAGO N-1E, Japan) measurement range of 0-32^{O}Brix

3.3.18 200 ml and 500 ml volumetric flasks

3.3.19 A Brookfield viscometer (Brookfield, England)

3.3.20 A colorimeter (Minolta, Japan)

3.3.22 A light microscope (Olympus, Japan)

3.3.23 A vernier caliper (VIS, Poland)

3.3.24 A centrifuge (Sanyo HARRIE 18180, Japan).

3.3.25 A colony counter (American optical company, USA)

3.3.26 250 ml and 500 ml glass bottles (DURAN®, Germany)

3.3.27 A hot air oven (Binder, UK)

3.3.28 An incubator at 37^{O}C (Gallenkamp, England)

3.3.29 A laminar air flow cabinet (Laminar ModelCF43S, Australia)

3.3.30 Sterile plastic petri dishes

3.3.31 A stomacher (Seward, England).

3.3.32 A vortex mixer (Gemmy Industrial, Taiwan).

3.3.33 An analytical balance (Mettler, Switzerland).

3.3.34 An analytical balance (Ohaus, USA).

3.3.35 An electronic juice blender (Central Industrial Supply Co., Ltd., Thailand)

3.3.36 A cold room storage at $4^{o}C$

3.3.37 Needle 0.40 x 12 mm 27G (Nipro, Japan).

3.3.38 10 ml syringe (Nipro, Japan).

3.3.39 1000 ml white bottles

3.3.40 Test tubes (5 and 10 ml) (Pyrex, USA).

3.3.41 Filter papers no. 40 (Whatman, Egland).

3.4 Chemical reagent

3.4.1 Calcium chloride (Merck, Germany)

3.4.2 Distilled water

3.4.3 Hydrochloric acid (HCl) (Merck, Germany)

3.4.4 Lactic acid (Fluka, UK)

3.4.5 Phenolphthalein (BHD, England)

3.4.6 Potassium hydroxide (KOH) (Merck, Germany)

3.4.7 Sodium hydroxide (NaOH) Merck, Germany)

3.4.8 Peptone water (Merck, Germany)

3.4.9 Phosphate buffer (Merk, Germany)

3.4.10 Sodium alginate (Fluka, UK)

3.4.11 Sodium chloride (NaCl) (J.T. Baker Mexico)

3.4.12 Bile-salt powder (Sigma, UK)

3.5 Microbiological media

3.5.1 de Man, Rogosa and Sharpe (MRS) Agar (Merck, Germany)

3.5.2 Potato dextrose agar (PDA) media (Merck, Germany)

3.5.3 Plate count agar (PCA) (Merck, Germany)

3.5.4 de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany)

3.6 Methods

3.6.1 Preparation and maintenance of *Lactobacillus acidophilus* TISTR 450

L. acidophilus TISTR 450 was grown anaerobically at 37°C for 48 hours and maintained in MRS agar as a stock culture. The stock culture of *L. acidophilus* was refreshed every 2 weeks during the research period. The strain of *L. acidophilus* was grown once in MRS broth from the stock culture for 16 to 24 hours at 37°C before used in an experiment. After this incubation, viable cells were harvested by centrifugation (4,300 x *g* for 10 minutes at 4°C) and washed two times in 0.85% sterile saline (Chou and Weimer, 1999).

3.6.2 Preparation of bean milk

Dried beans were soaked in distilled water with a ratio of 1:3 for 8 hours (Shurtleff and Aoyagi, 1984) and cleaned it twice with distilled water. The cleaned beans were then grinded and extracted with distilled water using a ratio of 1:5 (Shurtleff and Aoyagi, 1984) in an electronic juice blender. The liquid that was

extracted by the juice blender was recognized as bean milk. Afterwards, the collected bean milk was separated into 500 ml, filled into sterile glass bottles and pasteurized at 69 ± 3°C for 30 minutes (Marshall and Arbuckle, 1996) (the heating time for 500 ml bean milk was around 15-20 minutes). The pasteurized bean milk were cooled down and used in an experiment.

3.6.3 Study the effect of different types of bean milk on the survival of *L. acidophilus* during storage in bean milk at 4°C for 15 days

Bean milk solutions from 4 different types of beans, which were soy bean, black bean, mung bean and red kidney bean, were prepared according to the section 3.6.2. Into an amount of 500 ml of each pasteurized bean milk, a *L. acidophilus* culture with an approximate concentration of $1x10^9$CFU/ml was added and mixed homogeneously. The homogeneous solutions of bean milk were then aseptically separated into 50 ml sterile test tubes in a laminar air flow cabinet and stored at 4°C for 15 days. During the storage period, bean milk samples on 0, 3, 6, 9, 12 and 15 days of storage were taken and analyzed to monitor chemical, microbial and physical changes in the bean milk.

3.6.3.1 Analysis for chemical composition of different types of bean milk

1) Ash (AOAC, 2000)
2) Carbohydrate (AOAC, 2000)
3) Fiber (AOAC, 2000)
4) Lipid (AOAC, 2000)

5) Moisture content (AOAC, 2000)

6) Protein (AOAC, 2000)

3.6.3.2 For chemical changes of bean milk during storage at 4°C, several analyses were carried out. These analyses included:

(1) Total acidities (AOAC, 2000)

Ten milliliter of each bean milk sample was taken into a 125 ml erlenmeyer flask. After adding three drops of phenolphthalein as an indicator, the sample was titrated with 0.1M NaOH until the solution color appeared to be pink. Results were expressed as percentage of lactic acid (lactic acid /100 ml sample). The measurement of total acidities was done every 3 days during 15 days storage at 4°C.

$$\% \text{ lactic acid} = \frac{\text{ml of 0.1 M NaOH} \times 0.1 \times 0.009 \times 100}{\text{Volume of sample}}$$

(2) pH values by a pH meter

A volume of 10 ml bean milk samples was transferred into a 25 ml beaker. Into this sample, a pH meter probe was immersed for 5-8 minutes until the pH meter showed a constant pH value. The pH value shown by the pH meter was recorded. Prior to the measurement of bean milk samples, the pH meter was calibrated using 2 standard buffer solutions, which were pH 4 and pH 7. The measurement of bean milk

pH values was conducted every 3 days during 15 days storage at 4^{O}C and determined at room temperature.

(3) Total soluble solid by a refractometer

The total soluble solid of bean milk samples was measured with a hand refractometer and reported as ^{O}Brix. The measurement of the total soluble solid was conducted every 3 days during 15 days storage at 4^{O}C.

3.6.3.3 For physical changes of bean milk during storage at 4°C, several analyses were carried out. These analyses included:

(1) Viscosity by a Brookfield viscometer

(2) Color analysis by a colorimeter

3.6.3.4 Microbiological analysis

(1) Viable counts of *L. acidophilus* using MRS Agar (Dave and Shah, 1996)

L. acidophilus count from triplicate samples of each bean milk treatment was assessed every 3 days interval during 15 days storage at 4^{O}C. Each bean milk sample was mixed thoroughly and a sample amount of 1 ml of each milk sample was 10 fold serially diluted (10^5 to 10^9) in 0.15% peptone water. Enumeration was carried out using a drop plate technique (เรณู, 2543) on MRS medium. Plates were incubated anaerobically at 37^{O}C for 48 hours. Drops of sample containing 5 to 50 colonies were

enumerated, calculated as colony forming units (CFU) per ml and adjusted to log CFU/ml. The highest survival number of *L. acidophilus* in any experimental treatment within a specific experimental section was considered to be a suitable treatment that could maintain the viability of the probiotic bacterium in bean milk during storage at $4^{O}C$ and this experimental treatment would be further studied in the next experimental section.

(2) Total plate count for general viable microorganisms in bean milk using PCA medium (เรณู, 2543)

Total bacteria counted from triplicate samples of each bean milk treatment were monitored at 3 days interval during 15 days storage at $4^{O}C$. Each bean milk sample was mixed thoroughly and a representative sample of 1 ml from each milk sample was 10 fold serially diluted (10^5 to 10^9) in 0.15% peptone water. Enumeration was carried out using a drop plate technique (เรณู, 2543) on PCA medium. Plates were incubated aerobically at $37^{O}C$ for 72 hours. Plates containing 5 to 50 colonies were enumerated, recorded as colony forming units (CFU) per ml and calculated to log CFU/ml.

3.6.4 The effects of pH values and casein concentrations on the survival of *L. acidophilus* in bean milk during 15 days storage at $4^{o}C$

One type of bean milk solutions from the section 3.6.3 was chosen and used in this section. The chosen bean milk was prepared according to the section 3.6.2. However before the bean milk was pasteurized, the pH levels of the bean milk were varied into

3 different levels by adding 0.1M sodium hydroxide. The studied pH levels of the bean milk were at 6.0, 6.5 and 7.0. At the same time, the bean milk was added with 3 levels of casein, which were 0, 125 and 250 mg of casein/l. For the 9 treatments of the bean milk, each treatment was mixed homogeneously and pasteurized at 69 $\pm$ 3°C for 30 minutes (Marshall and Arbuckle, 1996). After cooling down the pasteurized bean milk, into each of 500 ml bean milk, a *L. acidophilus* culture with an approximate concentration of $1x10^9$ CFU/ml was aseptically added and mixed properly. Each treatment of the bean milk was then aseptically separated into 50 ml sterile test tubes and stored at 4°C for 15 days. During the storage period, bean milk samples on 0, 3, 6, 9, 12 and 15 days were taken and analyzed.

The chemical, physical and microbial analyses of this section followed the same procedure analyses as in the section 3.6.3. To monitor the extra addition of protein. the protein content of the bean milk samples was also analyzed weekly using an AOAC method (AOAC, 2000) and measured by a Kjeldahl digestion and distillation apparatus (Tecator, USA).

3.6.5 Investigation of an immobilized technique (an extrusion method) and initial concentrations of *L. acidophilus* in supporting the survival of the probiotic bacterium during storage in bean milk at 4°C for 15 days

3.6.5.1 Preparation of immobilized *L. acidophilus* cells

A 5 ml solution of *L. acidophilus* with a concentration of either $1x10^9$ cfu/ml or $1x10^{12}$cfu/ml was added into 45 ml 2% (w/v) alginate. The bacteria-alginate slurry

was allowed to mix thoroughly for 30 minutes using a magnetic stirrer at a speed of 5. With a 1 ml sterile syringe (0.5 mm gauge), 50 ml of the slurry was added dropwise into a beaker containing 0.1M calcium chloride. After keeping the beads at 4°C overnight in $CaCl_2$ for further hardening, the calcium chloride solution was decanted and the beads were washed with 0.85% sterile saline. All the washed beads originating from 50 ml of the slurry were treated as an inoculum. The entire process was carried out aseptically in a laminar flow chamber (Talwalkar and Kailasapathy, 2003).

The immobilized *L. acidophilus* cells were analyzed for the dimension of the beads and the efficacy of cells release from the beads (Talwalkar and Kailasapathy, 2003).

(1) Examination of alginate bead dimension

The dimensions of 108 randomly selected beads were determined using a vernier caliper.

(2) Examination of bead density

Bead density was calculated by the following formula

Density of bead = mass/volume

Volume of sphere = $(4/3)\ \pi\ r^3$

(3) Efficiency of cell release from the beads

For quantitative measurements of *L. acidophilus* viable cells by a drop plate method, it was necessary to solubilize the polymer beads to release the entrapped cells. To do this, one gram alginate beads was suspended in 9 ml of sterile 0.1M phosphate buffer at pH 7, followed by homogenizing in a stomacher for 5 minutes

(Krasaekoopt *et al.*, 2004). After doing dilutions with appropriate dilution factors, the colony forming units (CFU/ml) of *L. acidophilus* were determined by the drop plate method on MRS agar plates and incubated anaerobically at 37°C for 48 hours.

3.6.5.2 Study the effects of an extrusion method and initial concentrations of *L. acidophilus* on the survival of the probiotic microorganism in bean milk during storage at 4°C for 15 days

One type of bean milk from the results in the section 3.6.3 was prepared according to the preparation procedure in the section 3.6.2 and adjusted for its pH value and casein concentration following the results in the section 3.6.4. After a pasteurization process at 69 ± 3°C for 30 minutes (Marshall and Arbuckle, 1996), the bean milk was divided into 4 batches each contained 500 ml bean milk. Into the first 2 batches of the bean milk, free *L. acidophilus* cells with an initial concentration of either $1x10^8$cfu/ml or $1x10^{11}$cfu/ml were added and mixed properly. For the other 2 batches of the bean milk, encapsulated/immobilized *L. acidophilus* cells at an initial concentration of either $1x10^8$cfu/ml or $1x10^{11}$cfu/ml were incorporated and shaken thoroughly. The 4 batches of properly mixed bean milk were then aseptically separated into 50 ml sterile test tubes and stored at 4°C for 15 days. Bean milk samples were separated on 0, 3, 6, 9, 12 and 15 days storage to be analyzed according to the chemical and physical analyses as in the section 3.6.3. For microbiological analysis of the bean milk with encapsulated *L. acidophilus* cells, 1 ml of the bean milk sample with *L. acidophilus* beads was suspended in 9 ml of 0.1M phosphate buffer at pH 7, followed by homogenizing in a stomacher for 5 minutes (Krasaekoopt *et al.*, 2004). After releasing the *L. acidophilus* cells from the beads, the bean milk samples

together with the bean milk samples containing free cells of *L. acidophilus* were enumerated in different agar media following the microbiological analysis procedures in the section 3.6.3.

3.6.6 Evaluation for the survival of *L. acidophilus* in simulated gastrointestinal conditions during the shelf-life of the *L. acidophilus* in bean milk at 4°C for 15 days

3.6.6.1 Preparation of *L. acidophilus*-added bean milk

In this section, the best result from the section 3.6.5 was applied, particularly for one initial concentration of *L. acidophilus*. In the case of the section 3.6.5 showed immobilized cells of *L. acidophilus* had a better performance than that of the free cells, the free cells of *L. acidophilus* would be used in this section as a control. Bean milk was prepared according to the production method in the section 3.6.2 and the results in the sections 3.6.3 and 3.6.4. One concentration of *L. acidophilus* based on the results in the section 3.6.5 was added after the bean milk was pasteurized at 69 ± 3°C for 30 minutes (Marshall and Arbuckle, 1996). The *L. acidophilus*-added bean milk that contained either free or encapsulated cells of *L. acidophilus* was then aseptically separated into 50 ml sterile test tubes and stored in a refrigerator (at 4°C) for 2 weeks period.

3.6.6.2 The survival of *L. acidophilus* in a simulated high-acid gastric condition during the shelf-life of the *L. acidophilus* in bean milk at 4°C for 15 days

On the 0, 7 and 14 days of the *L. acidophilus*-added bean milk storage, samples of the bean milk were separated. Each of the bean milk sample was centrifuged at 4,300 x *g* for 10 minutes at 4°C to collect the microorganisms in the milk sample. After separating the liquid phase, the microorganism cells were subjected into MRS broths with different pH values. These pH levels of the MRS broth were 1.5, 2.0, 2.5 and 6.0 (6.0 for control). The tubes containing microorganism cells in the MRS broth were then incubated aerobically at 37°C for 3 hours. During the incubation time, sampling of microorganisms was conducted on 0, 1, 2 and 3 hours and drop plated on MRS agar to understand the survival of *L. acidophilus* in a simulated gastric acid condition (Hou *et al.*, 2003).

3.6.6.3 The survival of *L. acidophilus* in a simulated bile-salt condition during the shelf-life of the *L. acidophilus* in bean milk at 4°C for 15 days

Similar to the section 3.6.6.2, samples of the *L. acidophilus*-added bean milk were separated on 0, 7 and 14 days storage of the milks. The microorganisms in the bean milk were set apart by centrifuging the sample at 4,300 x *g* for 10 minutes at 4°C. The collected microorganisms were then subjected into three tubes of MRS broth that contained either 0, 1 or 2% (w/v) bile-salt concentrations. Following the microorganism inoculation, the tubes of the microorganism cells in the MRS broth containing bile-salt were aerobically incubated at 37°C for 3 hours. Samples of microorganisms were collected on the 0, 1, 2 and 3 hours of the incubation time and drop plated on MRS agar to study the survival of *L. acidophilus* under a simulated bile-salt condition (Hou *et al.*, 2003).

3.7 Experimental designs and statistical analysis

Collected data from the experimental section 3.6.3 was statistically analyzed by Analysis of Variance using a randomized completely block design with three replications. For the experimental sections 3.6.4, 3.6.5 and 3.6.6, the data was statistically analyzed by Analysis of Variance using a Factorial Experiment in completely randomized design with three replications. To determine differences between treatment means, a Least Significant Difference (LSD) test and Duncan were employed (พิสมัย, 2547). All of the statistical analysis was conducted in the SPSS statistical software version 10 (SPSS Inc., U.S.A).