## Chapter III

## Research Methodology

### 3.1 Equipment and Chemical substances

**Equipment:**

- Microscope, NIKON
- Incubator, BINDER
- Analytical Balance; 4 digits, CP 22 4S/SARTOURIUS
- UV-Vis Spectrophotometer, HP HEWELLT PACKARD
- Microbiological Cabinet, MARK II /DWYER
- Hot Plate/ Magnetic Stirrer, WSAH2/WELLAB MODEL
- Autoclave, HVE-50/HIRAYAMA, andLABO/SUNYO
- pH meter, EUTECH INSTRUMENTS
- Hot Air Oven, 100-800/OVER MEMRET
- Water Bath, MEMRET
- Vortex Mixture
- Test Tube
- Petri Dish or Petri Plate
- Glass Slide and Cover Glass
- Spreader
- Erlenmeyer Flask/Florence Flask
- Pipette/Auto- micropipette
- Stirring Rod
- Beaker
- Plastic MicrocentrifugeTube
- Micropipette Tip
- Test Tube Rack
- Aluminum Foil
- 0.6 mmDiameter Filter Paper
- Forceps
- Loop and Needle
- Cotton Wool

- 7 mmDiameter AluminumRing

**Culture medium**

- MRS Broth (de Man Rogasa& Sharpe, Lab Scan)
- MRS Agar (Lab Scan)
- Milk Agar (Difco)
- Starch Agar (Difco)
- Bile Salt V (Sigma)
- Tributyrin Agar (Sigma)
- CMC Agar (Merck, Germany)
- Tryptic Soy BrothandAgar (TSBandTSA; Difco)
- Nutrient Agar (NA, Difco)
- Brain Heart Infusion Agar (BHI; Merck)
- Sabouraud Dextrose Broth and Agar (SDBandSDA; Difco)
- API® 50CHL Medium (Biomerieux)
- Mueller Hinton Agar (MHA)
- Trypticase Soy Broth (TSB)
- Sabouraud Dextrose Agar (SDA)
- Sabouraud Dextrose Broth (SDB)

**Chemical substance**

- NaCl 0.85 % (Sodium Chloride 0.85%)
- Phosphate Buffer Saline (PBS, pH 7.2)
- Bromocresol Purple
- 1 N HCL (1 N Hydrochloric Acid)
- 1 N NaOH (1 N Sodium Hydroxide)
- Iodine Solution
- 3%Hydrogen Peroxide Solution
- Gram Stain
- 95% Alcohol
- Oil Immersion Lens
- Gentamicin (Antibiotic Control)
- Amphotericin B (Antibiotic Control)
- MacFarland No. 0.5

- Sterilizer

**Test Microorganisms**

- *Escherichia coli* ATCC 25922
- *Staphylococcus aureus* ATCC 25923
- *Pseudomonas aeruginosa* ATCC 27853
- *Bacillus cereus* ATCC 11778
- *Candida albicans* ATCC 90028

### 3.2 Research Methodology

Permission was granted for animal experimentation by the Animal Experimentation Ethics Committee, Faculty of Veterinary Medicine, ChiangMai University.

Sampling was conducted at Maesa Elephant Camp, Maerim District, Chiang Mai, Thailand, and research was done at the laboratory of the Faculty of Pharmacy, ChiangMai University, Suthep Subdistrict, Muang District, Chiang Mai, Thailand.

**Progress of the study**

1. Monograph and literature review
2. Screening of microorganisms from elephant feces

2.1 Elephant feces sampling

2.1.1 Twenty-seven captive Asian elephant feces at Maesa Elephant Camp, Maerim District, ChiangMai, Thailandwere collected for this study.

2.1.2 The elephants from which feces had been collected were varied in terms of gender and weight and were considered to be healthy during the sampling period. The following data were collected from each elephant:

Annual vermicide profile, basic data, category of usability, food intake, rations fed, type of feedstuff (continualor adjusted at least one week), and number of meals.To the best of the researchers' knowledge, none of theelephants had received antibiotics for the seven days prior to sampling.

2.1.3 A new pair of gloves and liquid paraffin were used for each direct rectum feces collection, or incase it could not be collected directly from the rectum

feces, samples were collected directly after falling to the ground. Approximately 25 g of samples were collected in a sterile close fitting container and transported to the laboratory within one hour.

2.1.4 Microbial isolation was performed by using the following media and incubated at appropriate conditions.

(i) Plate count agar (PCA)

(ii) Potato Dextrose Agar (PDA), and

(iii) MRS Agar.

Colonies that could grow in these media were then tested for probiotic properties.

3. Laboratory identification of microorganisms

Bacteria from discrete colonies of confirmed probioticswere tested for the following properties:

*3.1 Bile salt tolerance test* (method adapted from Conway et al., 1987; Toit et al., 1998; Chou and Weimer, 1999)

Bile salt tolerance was tested by streaking the bacteria in an MRS agar plate with 0.30 (w/v) bile concentrations. All the plates were incubated at 37 °C for 24-48 h. Samples were analyzed in triplicate. Bile tolerance was determined by checking for the viable cells on the MRS agar containing bile salt.

*3.2 pH tolerance test* (method adapted from Toit et al., 1998; Chou and Weimer, 1999; Halm et al., 2004).

Study of the acid-base tolerance of the lactic acid bacteria was performed in 5 ml MRS broth. Hence, the pH of the broth was adjusted to 2, 3, 4, 5, 8, and 9, respectively, using 1 N HCL or 1 N NaOH. Each assay was performed in triplicate. These broths were then incubated at 37 °C for 24 h. Acid-base tolerance was determined by turbidity of the broth detected by spectrophotometry $OD_{660}$ ($\lambda$=660) at 0 h and after 24 h of incubation.

*3.3 Cellulolytic properties* (adapted from Teather and Wood, 1982 and Baharuddin et.al, 2010)

The detection of cellulolytic properties was performed using 1% (w/v) Congo red poured on carboxymethyl cellulose (CMC) agar containing test organism,

incubated for 15 min, followed by rinsing using 1M NaCl. Incubation for 15 min again, and washed out. Congo red stain of the CMC reacted with Polysaccharides ß-(1-4)–linked D-Glucopyranosyl unit, β-(1-3)-D-glucans,and hemicellulosicgalacto-glucomannans. This activity was detected by the appearance of clear zones.

*3.4 Carbohydrate, protein and lipid utilization* (method adapted from Duangjitcharoen, et al., 2008)

*Carbohydrate utilization property*

The assays were made by streaking lactic acid bacteria, which had been growing in MRS broth for 18 h, on the Starch agar plate. The tests were made in triplicate, then incubated at 37 °C for 48 h. The carbohydrate utilization property was indicated by dropping the iodine solution on the agar plate; if the carbohydrates were utilized, the iodine solution would not turn blue.

*Protein utilization property*

Milk Agar was used to determine the utilization of protein by streaking lactic acid bacteria, which has been growing in MRS broth for 18 h. Then it was incubated at 37°C for 48-72 h. The test was made in triplicate. Any clear zone around bacterial colonies in each agar medium indicated that the microorganisms had utilized the protein substrates.

*Lipid Utilization property*

The lipid utilization property ofmicroorganism was determined by streaking the lactic acid bacteria, which has been growing in MRS broth for 18 h, on the 1% Tributyrin agar plate. The tests were made triplicate, then incubated at 37 °C for 48-72 h. Any clear zone around bacterial colonies in each agar medium indicated that the microorganism had utilized the lipid substrates.



*3.5 Aerobic and anaerobic viability test*

Two groups of MRS agar plates were streaked with bacteria from discrete colonies of confirmed probiotics. The first group of plates was incubated in a 5% $CO_2$ incubator andanother group of plates were incubated in an aerobic condition. Both were incubated at 35-37 °C for 2-3 days.

*3.6 Pathogen inhibiting property* (method adapted from Sililun, 2005)

1. The isolate cultured brothwas filtered through a 0.22 µm membrane filter into clean, sterile test tubes.

2. The supertnatants were tested by agar cup plate for antimicrobial activities that inhibit the growth of the following test microorganisms:

- *Escherichia coli* ATCC 25922
- *Staphylococcus aureus* ATCC 25923
- *Pseudomonas aeruginosa* ATCC 27853
- *Bacillus cereus* ATCC11778, and
- *Candida albicans* ATCC 90028

3. The test microorganisms were a culture in Trypticase soy broth (TSB) and Sabouraud dextrose broth (SDB) for 2-3 h. Then a turbidimetric comparison of the test culture was conducted with a control culture, Mac Farland No. 0.5. (Equal to Turbidity of bacteria and yeast of $10^8$ and $10^6$ CFU/ml, respectively)

4. The known turbidity test microorganisms were diluted to 1:100, and 1 ml of the diluted test microorganisms was inoculated in melted MHA and SDA medium. Mediums were melted at 45-50°C. The well-mixed broth was filled into the petri dish as a first layer. The process was made in duplicate.

5. After the medium had been set, forceps were used to set 4-5 sterile aluminum rings down in each medium.

6. The turbidity adjusted test bacteria and yeast (1 ml) were added into melted MHA and SDA, shaken well, then poured on the culture plate as the second layer.

7. After the medium set, forceps were used to pull the sterile aluminum rings out from the hardened medium. The sterile aluminum rings were then soaked in the sterilizer. After this process, wells for test samples were punched in the medium.

8. One hundred µl of filtered cell free supernatant were poured in each well.Then Amphotericin B was added to the yeast plate, and Gentamicin was added to the bacteria plate as a positive control.

9. The plates were incubated at 35-37 °C for 18-24 hours, and the diameter of the clear zone around the reservoir (inhibition diameter), including the diameter of aluminum ring, was measured at the end of the incubation period.

*3.7 Growth curve*

The dynamics of the bacterial growth can be studied by plotting cell growth (Measured as turbidity at $OD_{660}$) versus the incubation times at 0, 3, 6, 9, 12, 15, 18, 24, 36, and 48 h.Thus we can obtain a sigmoid curve and is known as growth curve.

4. Bacterial strain identification by biochemical test and 16S rRNA Gene Sequencing.

4.1 Sugar Utilization Kit API50CHL (Sirilun, 2005; Sirilun et.al, 2010)

Activelygrowing bacterial cultures (49 strains)were examined according to the instructions for the use of API 50 CHL to identify carbohydrate metabolism and bile esculin.Prior to examination with API 50 CHL, strains were cultivated in MRS broth at 35°C-37°C for 10-18 h. The test kit was inoculated with cell suspension with a turbidity equivalent to Mc Farland concentration of $10^8$ CFU/ml. The results were evaluated using API 50 CHL Medium (Biomerieux) compare program.

4.2 Identificationof Bacteria by partial 16s RNA gene sequence

Actively growing bacterial cells were sub-cultured in MSR agar and sent to the KU-Vector, Custom DNA Synthesis Service Unit at Kasetsart University for the strain identification.

5. Collection and conclusion of laboratory results

6. Report, analysis of results and summary