CHAPTER III
MATERIALS AND METHODS

1. Sampling, Enumeration and Isolation of the Microorganisms Derived from Mhom Produced Locally in the Northeastern of Thailand

1.1 Sampling procedure

5 Mhom samples produced from meat and sold at different local markets in four provinces, such as Khon Kaen, Mahasarakham, Chaiyaphum and Kalasin, located in the Northeastern region of Thailand. Samples were collected at 20-to-30-day-ripening period and directly transported to the laboratory at Department of Food Technology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand. Specimens were kept under refrigeration (below 4 °C) for further chemical and microbiological analyses.

1.2 Chemical analysis

Total acidity and pH values were determined using the methods established in the previous report of Franco et al. (2002). Analysis of such parameters was carried out in triplicate for each sample. Means and standard deviations were calculated.

1.3 Microbiological analysis

All samples were microbiologicaly analyzed and counted for (1) Aerobic plate counts; (2) Lactic acid bacteria (LAB); (3) Micrococcaceae; (4) Coliforms; (5) Enterococci; (6) *Escherichia coli* O157:H7; (7) *Staphylococcus aureus*; and (8) Yeasts and Molds as previously described (Fontana et al., 2005)

Each Mhom sample (10 g) was aseptically transferred to the sterile stomacher bag. 90 ml of Saline-Peptone water (2% NaCl, 0.1% bacteriological peptone, 1% Tween-80) was then added. The preparation was mixed for 3 min with a stomacher machine. Additional decimal dilutions were prepared, and the following analyses were proportionally performed, i.e., (1) Aerobic plate counts from Plate Count agar incubated for 48 h at 30°C; (2) LAB counts from MRS agar incubated for 48 h at 30°C in an anaerobic jar under restricted oxygen condition;

(3) Micrococcaceae counts from Mannitol Salt agar (MSA) incubated for 48 h at 30°C; (4) Coliforms counts from MacConkey agar incubated for 24 h at 37°C; (5) Enterococci counts from *Streptococcus faecalis* (SF) agar incubated for 72 h at 42°C; (6) *Escherichia coli* O157:H7 from Sorbitol MacConkey Agar (SMAC) incubated for 24 h at 37°C; (7) *Staphylococcus aureus* counts from Baird-Parker medium containing egg yolk tellurite emulsion incubated at 37°C for 24 to 48 h; and (8) Yeast and Mold counts from Sabouraud agar incubated at 30°C for 48 to 72 h. Three independent measurements were carried out for each sample. After counting was accomplished, the values of mean and standard deviation of each sample were calculated.

Colonies of LAB (10) were randomly selected from the MRS agar plates of each Mhom sample, transferred to MRS and BHI (Brain hearth infusion) broth, and incubated overnight at the appropriate temperature. Colonies were maintained in the same liquid media containing 30% glycerol and kept at –20°C for further analyses. All isolates were preliminarily characterized by means of cell morphology and Gram stain, and tested for the catalase reaction.

2. Biochemical, Molecular Characterization and Identification of the LAB Isolated From Mhom Produced Locally in the Northeastern of Thailand

2.1 Characterization and identification of the LAB isolates utilizing the carbohydrate fermentation profiles derived from the API 50 CH strips and API CHL medium

Colonies (5) of LAB isolates were randomly selected from the MRS agar plates of each Mhom sample. The colonies were carefully tested and purified on the MRS medium. The pure cultures were first characterized by using the Gram stain and cell morphology. Gram-positive and catalase-negative isolates were stored at –80°C in MRS broth containing 30% glycerol (70/30, v/v; Merck, Darmstadt, Germany) until used and for further identification by molecular methods. The isolates were subjected to two transfers in MRS broth for activation before use. The isolates were identified at the species level according to the methodology and defined characteristics given by Curk, Hubert, and Bringel (1996). The carbohydrate

fermentation profiles of the selected isolates of all Mhom samples were investigated utilizing the API 50 CH strips and API CHL medium according to instructions of the manufacturer (API system, Bio-Merieux, France) (Ammor et al., 2005).

2.2 Molecular characterization and identification of the isolated LAB by using RAPD-PCR

Isolated LAB cells were subcultured overnight on agar plates at 32 °C. Single bacterial cells inoculated in broth (TSB with 0.5% Yeast Extract) at 32 °C for overnight, pipette 1.5 ml of broth culture into 2 ml microtube, pelleted cells washed by 1.5 ml of saline (0.85 % NaCl), and then centrifuged at 13,000 rpm for 2 min. Washed cells dissolved in 20 µl of saline (Sambrook and Russell, 2001).

The RAPD-PCR assay was performed as described previously (Fontana *et al.*, 2005) by using the GeneAmp PCR System 9600 thermocycler (Applied Biosystems, USA). Each RAPD-PCR reaction was carried out in a total volume of 50 µl containing 1x PCR buffer, 25 mM $MgCl_2$, 100 µM dNTP of each, 0.3 µM of each primer, i.e., LMPB1 5′-GGAACTGCTA-3′ and LMPB4 5′-AAGGATCAGC-3′ (Boerlin et al., 1995), 1.25 units Taq DNA polymerase, 5 µl of suspension cells, then adjusted dH_2O to a final volume 50 µl. All reagents were added together (except for the bacterial cells) into the reaction vial and mixed thoroughly. In the initialization step, the samples were initially incubated for 6.5 min at 94°C and lyse cells hot stand PCR subsequently, samples were amplified for 35 cycles. Each cycle consists of a denaturation step of 1 min at 94°C, an annealing step of 2 min at 35 °C, an extension step of 1 min at 72 °C, and finally an elongation step of 10 min at 72°C to ensure that any remaining single-stranded DNA was fully extended. When the PCR was completed, the RAPD-PCR products were electrophoresed at 100 V on 2.5% agarose gels and stained with ethidium bromide as described previously (Fontana *et al.*, 2005). The RAPD–PCR profiles were analyzed utilizing the NTSYS-PC software package and a dendrogram was generated by using the UPGMA program.

2.3 Characterization and identification of LAB by using 16S rDNA gene sequencing

Chromosomal DNA of LAB was extracted with DNA extraction kit and according to the manufacturer's instruction. A 345-bp fragment of the 16S rDNA gene of the bacterial isolates was amplified by
LABF (5'-ACGGGAGGCAGCAGTAGGGA-3') and
LABR(5'-CGCTACACATGGAGTTCCAC-3') Each RAPD-PCR reaction was carried out in a total volume of 50 µl containing 1x PCR buffer, 25 mM $MgCl_2$, 100 µM dNTP of each, 0.3 µM of each primer, 1.25 units Taq DNA polymerase, approximately 10 ng of genomic DNA and adjusted dH_2O to a final volume 50 µl. Initial heating at 97 °C for 4 min, was followed by 40 cycles of the following sequence: 97°C for 2min, 65 °C for 4 min, 80 °C for 2 min. DNA sequencing was continually performed by Biotechnology Resource Center, (DNA services), New York, USA. (Fontana et al., 2005, Klijn et al., 1991, Mellmann et al., 2003, Muyzer et al., 1993, Woo et al., 2003).

3. Screening and Application of LAB as the Starter Cultures for Mhom Production

3.1 Microbiological quality of raw materials and ingredients

Raw materials and ingredients, such as unpeeled and peeled garlic, beef, liver and spleen, were evaluated for their microbiological quality, including aerobic plate counts, and counts of LAB, Micrococcaceae, Coliforms, Enterococci, *Escherichia coli* O157: H7, *Staphylococcus aureus*, yeasts and molds as previously described (Fontana et al., 2005, Rogosa et al., 1951).

3.2 Screening of the already identified LAB

The already identified LAB were screened and would be used for starter application through the methods listed as follow:

3.2.1 Primary screening of LAB

The primary screening for antimicrobial activity against several pathogenic microorganisms and hydrogen peroxide production by LAB were determined as described previously (Gibello et al., 1999)

3.2.1.1 Determination of antimicrobial activity of LAB against several pathogens

1) Bacterial indicator strains and growth conditions

Three strains of *Staphylococcus aureus*, such as ATCC13565, ATCC25923 and ATCC25904, *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Pseudomonas* sp. were kindly provided by the Food Microbiology Laboratory, Faculty of Technology, Mahasarakham University, Thailand and were employed as the indicator strains. Testing for the antimicrobial activity of LAB was carried out at the Food Microbiology Laboratory, Department of Food Technology and Nutrition, Faculty of Technology, Mahasarakham University, Thailand.

The pathogenic bacterial stock cultures were stored frozen in 40% (v/v) glycerol-ether nutrient broth. Working bacterial cultures were grown at 37°C for 24 h on the nutrient agar. To obtain the bacterial cells at the stationary growth phase, bacterial cultures were subcultured and incubated twice at 37°C for 24 h in nutrient broth. Cells were harvested by centrifugation at 6,000 g for 2 min and washed once with a 5 mM NaCl solution. The supernatant was discarded and the pellet containing bacterial cells was washed again. Bacterial cells were harvested and suspended in the freshly prepared nutrient broth. The optimum concentration of cultures was 10^6 colony forming units/ml, i.e., 1×10^6 CFU/ml.

2) Detection of antimicrobial activity of LAB in broth culture

The antimicrobial activity of the LAB isolates was determined by modifying the disc diffusion method of Hamadan and Mikolajcik, (1974). Cell-free supernatants of LAB were obtained from the LAB cultures propagated in MRS broth for 24 h at 30°C under anaerobic conditions. The LAB cell pellets were spun down, and the supernatants were collected and adjusted to pH 6.5 with 10N NaOH, and sterilized by filtering through a 0.22 mm-pore-size cellulose acetate filter (Altech Associates, Inc. 2051 Waukagen Road, Baerfleld, IL, 60015, USA). Sterile filter discs (6 mm) were dipped into the LAB cell-free supernatants and incubated at 30°C for 42 h, and placed on the Muller-Hinton agar (OXOID) plates

seeded with 12 to 14 h cultures of the test microorganisms. The plates were kept at 4°C for 3 to 4 h to permit the diffusion on the assay material, and then incubated at 37°C for 14 to 16 h. The sterile filter discs dipped in un-inoculated MRS broth served as the controls. The clear zones were detected, and their diameters were measured with the verneir caliper. The antibiotic activity tests were done in duplicates and the mean values were evaluated and recorded.

3.2.1.2 Lactic acid productivity of the LAB isolates

The amount of lactic acid produced in the cell-free supernatants of the LAB isolates was quantified by high performance liquid chromatography (HPLC) according to the method of Butkhup and Samappito (2008). An HPLC apparatus consisting of a Shimadzu LC–20AD Series pumping system, SIL–10AD Series Auto injector system and SPD–M20A Series Diode array detector (Shimadzu Cooperation Analytical and Measuring Instruments Division, Kyoto, Japan) was used to record the online UV spectra of the organic acids in the samples. Data were collected and analyzed with the Shimadzu computing system. The column used was an Apollo C_{18} (Alltech) (ø 4.6 mm x 250 mm, 5 µm) protected with a guard column Intertsil ODS–3 (4.0 mm, 10 mm, 5 µm). Each sample (20 µl) was analyzed using the HPLC-UV detection system. Elution was effected using an isocratic elution of the solvent, 25 mM phosphate buffer (Ajax) (pH 2.5) at a flow rate of 0.9 ml/min and column temperature of 40°C. The UV–Vis spectra were recorded from 190 to 400 nm, with detection at 210 nm. L–lactic acid (Fluka) solutions with the concentrations ranging from 0.5 to 25 g/l were used as the external standards to produce the standard curve (lactic acid concentration versus peak area) used for the quantitative analysis of the lactic acid produced by the samples.

3.2.2 *Secondary screening of LAB*

Physiological characteristics, growth and acidifying capacity were evaluated as previously described (Buckenuskes, 1993; Holzapfel, 2002).

3.2.2.1 Tolerance of LAB to high temperature, high concentration of lactic acid and sodium chloride and low pH

The basal MRS medium constituted without beef extract and supplemented with bromocresol purple as the pH indicator of 7.0 at the

concentration of 0.17 g/l was used in the present study. A lowered pH would change the color of the medium from purple to yellow, and this phenomenon was taken to indicate the growth rate of LAB cells because the production of lactic acid is growth-related. No change in the color of the medium was included to indicate no cell growth because no acids were produced. Test tubes with screw caps were each filled with 20 ml of the basal MRS medium and autoclaved for sterilization. Twenty-four hour cultures of each isolate were used as the inocula, whereby the cells were spun down, suspended in 0.85% saline, and 50 μl of the suspension was inoculated into each test tube. The temperatures evaluated were 15, 30, 37, 45 and 50°C. The lactic acid concentrations were 2.5%, 5%, 7.5%, 10% and 15% (w/v). The concentrations of NaCl evaluated were 1.5%, 2.5%, 5%, 7.5% and 10% (w/v). The pH values examined in this study were 2.5, 4.5, 7.0 and 9.0. In doing so, the basal MRS medium was adjusted with 1M phosphoric acid, or 1M NaOH. The test tubes were incubated in the water bath equipped with a temperature controlling system and set at the specific test temperatures or at 37 °C for the other tests of the tolerance to different pH values and concentrations of lactic acid and NaCl. At the end of 48 h, the changes in color and turbidity of the medium in each tube were noted as the simple indication of growth or no growth of LAB. The experiments were carried out in triplicate.

3.2.2.2 Growth and lactic acid production profiles of LAB during the time course study

Based on the screening test for the tolerance of LAB to various extreme environmental conditions, the five LAB isolates, i.e., CP116, CP120, CP210, MK114 and KS103 were selected to pursue the time-course study to generate their growth and lactic acid production profiles. 50 ml of the 24 h cultures was used as the inoculum whereby the cells were spun down and washed twice with 0.85% saline before being transferred into 1000 ml Erlenmeyer flasks containing 400 ml basal MRS medium (pH 7.0) which were prepared in triplicate for each isolate tested. The flasks were incubated at 37°C. The sampling was performed at every six-hour-interval for 54 hours. At each sampling time, 20 ml of the cultures were removed aseptically from each flask for pH determination of amount of lactic acid and residual glucose present. Each analysis was performed in triplicate for each sample.

3.2.2.3 pH measurement

The pH value were determined using the methods cited by Franco et al. (2002). In this experiment, all determination were made in triplicate with each sample.

3.2.2.4 Lactic acid assay

Samples of cultures (1 ml) were centrifuged at 14,000*g* for 10 min at 4°C. The supernatants were decimally diluted with ten-fold dilution factor using the ultra pure water. The amount of lactic acid in the diluted supernatants was analyzed with the HPLC-UV detection system as previously reported (Butkhup and Samappito, 2008) and described in 3.2.1.2.

3.2.2.5 Quantification of residual glucose

The ten-fold diluted supernatants as indicated in 3.2.2.4 were also used for the assay of residual glucose using dinitrosalicylic acid (DNS) (Miller, 1959). DNS reagent (3 ml) was added into the lightly capped tubes containing 3 ml of supernate samples. To avoid the loss of supernate due to evaporation, the tubes were covered with paraffin film if plain test tubes were used. The mixture were heated at 90° C for 5-15 min to develop the red-brown color. 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature using the cold water bath, the absorbance at 575 nm was obtained utilizing a spectrophotometer.

3.3 Application of LAB as the starter cultures

The already identified and selected LAB were assigned as different combinations of starter cultures, and were used to ferment the Mhom products.

3.3.1 Preparation of Mhom

The Thai traditional dry fermented sausages, "Mhom", were prepared according to the following formula: beef 300 g/kg, liver 400 g/kg, spleen 100 g/kg, roasted rice powder 85 g/kg, garlic 85 g/kg, salt 30 g/kg and glucose 3 g/kg. The minced mixture was divided into eight different batches, i.e., control or naturally fermented without the addition of starter cultures and batches II to VIII with different starter cultures as shown in Table 3.1.

Table 3.1 Starter cultures and their combination used in Mhom fermentation

Batch	Starter cultures
I	No starter cultures added (i.e., control)
II	*L. plantarum* 1 CP120
III	*L. plantarum* 1 CP210
IV	*L. plantarum* 2 MK114
V	*L. plantarum* 1 CP120 : *L. plantarum* 1 CP210 (1:1, v/v)
VI	*L. plantarum* 1 CP120 : *L. plantarum* 2 MK114 (1:1, v/v)
VII	*L. plantarum* 1 CP210 : *L. plantarum* 2 MK114 (1:1, v/v)
VIII	*L. plantarum* 1 CP120 : *L. plantarum* 1 CP210 : *L. plantarum* 2 MK114 (1:1:1, v/v/v)

The starter cultures were previously isolated, identified and selected from indigenous fermented Mhom based on their high antimicrobial activity and tolerance to extreme environmental conditions. Freeze-dried starter cultures of 2 strains of *L. plantarum* 1, i.e., CP 120 and CP 210, and 1 strain of *L. plantarum* 2 MK-114 were added at the levels of 10^6 colony forming units (CFU)/g of meat batter were used as inocula in the meat fermentation experiments (Mhom). Each batch of mixture was homogenized with the other ingredients using the mixer (TV-VALL, Amui-80, Spain) and the final mass of each batch was stuffed into the natural cow casings. Each sausage was weighed for 300 g and fermented for 28 days at 25°C (RH 75%).

3.3.2 Sampling procedure

Sampling was performed by randomly selecting two links of each Mhom preparation after 0, 1, 3, 5, 7, 9, 11, 14, 21 and 28 days of ripening period for analyses of the microbiological parameters, pH, total nitrogen (TN) and moisture content. Samples collected for such analyses were kept at -20°C before the time of analyses as previously recommended (Bover-Cid *et al.*, 1999).

3.3.3 Microbiological analysis

Sample of the core of Mhom (10 g) were aseptically removed, diluted with 90 ml of Saline-Peptone water (2% NaCl; 0.1% bacteriological peptone; 1% Tween-80) and homogenized in the Stomacher (LAB-BLENDER, 400, London,

UK). The homogenate was decimally diluted, i.e., 10-, 100-, 1000-, and even 10^4-, or 10^5-fold as if necessary. Then 0.1 ml of samples at the appropriate dilution were spread in duplicate on to the selective agar plates. Flora numbers were determined from plates bearing 25 to 250 colonies. The specific and different counts were obtained as follows: (1) Total aerobic plate counts on Plate Count agar (Difco, Detroit, Michigan, USA) incubated for 48 h at 30°C; (2) LAB counts on the MRS agar (Difco) incubated for 48 h at 30°C in an anaerobic jar under restricted oxygen condition; (3) Micrococcaceae counts on Mannitol-Salt agar (Difco) incubated for 48 h at 30°C; (4) Coliforms counts on MacConkey agar (Difco) incubated for 24 h at 37°C; (5) Enterococci counts on *Streptococcus faecalis* agar incubated for 72 h at 42°C; (6) *Escherichia coli* O157:H7 on Sorbitol MacConkey agar incubated for 24 h at 37°C; (7) *Staphylococcus aureus* counts on Baird-Parker agar containing egg yolk tellurite emulsion (Difco) incubated at 37°C for 24 to 48 h; and (8) Yeast and Mold counts on Sabouraud agar (Difco) incubated at 30°C for 48 to 72 h.

3.3.4 pH measurement

Each sample (5 g) were suspended with 5 ml distilled water. The mixture was mixed vigorously with the Sorvall Omnimixer homogenizer (Omni International, Waterbury, CT, USA). Measurement of the pH values of samples was performed with pH meter (ATI ORION 420 A, USA).

3.3.5 Moisture content

The moisture content of samples was determined according to the International Standards Organization ISO Method 1442 (1973). The moisture was determined by drying the sample at 100-105°C. (ISO R-1442)

3.3.6 Weight loss

Weight loss was determined as previously described (Nakao *et al.*, 1991). All Mhom samples with casing (100 g) were accurately weighed before fermentation by using a four digit balance (Model AG 204, Mettler Toledo). Then, after 1, 3, 5, 7, 9, 11, 14, 21 and 28 days of fermentation and ripening, Mhom samples were weighed. Difference in weight of Mhom samples before and after fermentation and ripening period was expressed as weight loss.

3.3.7 Lactic acid assay

The lactic acid produced in Mhom samples was analyzed with the HPLC-UV detection system as previously reported (Butkhup and Samappito, 2008) and described in 3.2.1.2.

3.3.8 Residual glucose assay

The ten-fold diluted supernatant as indicated in 3.2.2.4 was also used for the assay of residual glucose using dinitrosalicylic acid (DNS) as previously reported (Miller, 1959) and described in 3.2.2.5 .

4. Monitoring the Population Dynamics of the Appropriate Starter Cultures During the Fermentation Period

Several combinations of the appropriate starter cultures as shown in Table 3.1 were applied into the mixture of raw materials and ingredients. The microbial profiles were then followed and evaluated for the dynamic changes during the fermentation period (Fontana *et al.*, 2005). In addition, Mhom samples prepared with the defined combination of the appropriate starter cultures were determined for proximate analysis.