CHAPTER V
CONCLUSIONS

The present study consisted of four major parts: Part I: Sampling, enumeration and isolation of microorganisms derived from Mhom produced locally in Northeastern region of Thailand; Part II: Biochemical and molecular characterization and identification of LAB; Part III: Screening and application of LAB as the starter cultures for Mhom production, and Part IV: Monitoring the population dynamic of the appropriate starter cultures during the fermentation period. The results obtained in each part were concluded as follows:

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

In general, Mhom products are manufactured with traditional technologies without application of the pure starter cultures. Their manufacturing process depends upon the skill and experience of the manufacturer rather than being a process that is fully based on scientific and technological means. Hence, the process depends on the growth of autochthonous microflora which influences the flavor, texture, nutritional qualities, safety, and other characteristics of this type of sausage. The isolation and selection of lactic acid bacteria which can be used as starter cultures may help in obtaining Mhom products with reproducible hygiene and organoleptic qualities in a shorter ripening time.

In this part of study, Mhom samples manufactured locally in five different places of Northeast Thailand provinces, i.e., Chaiyaphum 1 (CP1), Chaiyaphum 2 (CP2), Khon Kaen (KK), Mahasarakham (MK), and Kalasin (KS) were followed to evaluate their physicochemical parameters, i.e., pH and total acidity, and to quantify and isolate microorganisms involved in the manufacturing process. The results were summarized as follows:

1. The average pH value was 4.7, and this value was within the range of the standard pH value of Mhom products provided and accepted by the Community

Product Standard No. 146/2546 issued by the Thai Industrial Standards Institute, Ministry of Industry, Thailand. This result indicated that Mhom samples with the average pH of 4.70 are safe for consumption.

2. With the similar time period of fermentation, i.e., 20 to 30 days, the average percentage of total acidity of lactic acid found in all Mhom samples was 1.06. However, an inverse relationship between the values of total acidity and pH was observed. The possible explanations could be the presence with inconsiderable amounts of other organic acids, such as acetic, butyric and succinic acids, or the growth and development of other micro flora, particularly yeasts and molds, as they are capable of consuming lactic acid in the presence of an organic nutrient for the bacteria.

3. The analysis of microbiological load of Mhom samples resulted in high total aerobic plate counts, yeast and mold counts, LAB count, *Micrococaceae* count and *Enterobacteriaceae* count. LAB were the main microbial groups. These results were similar to those observed in the other acid fermented meat sausages. *E. coli* O157:H7 was not detected in all Mhom samples. The high numbers of *S. aureus* indicated poor hygienic practices. Thus, it is recommended that Mhom produced locally in such provinces should be consumed as a cooked food. From the nutrition point of view, Mhom products have some valuable physicochemical composition, i.e., decreased pH and increased acidity, thereby preventing the growth and development of naturally occurring pathogenic bacteria.

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

For identification of LAB species isolated from Mhom samples, the biochemical profiles obtained from the API 50 CH carbohydrate fermentation strips and API 50 CHL medium, RAPD-PCR profile clusters obtained with LMPB1 and LMPB4 primers and sequencing analysis of the mitochondrial 16s rRNA gene, were employed in this study. A total of 71 LAB strains were isolated from MRS agar plates and subjected to biochemical, molecular identification and characterization by API-50 test followed by sequencing and RAPD-PCR with cluster analysis of the profiles. The results obtained highlighted that:

1. The main populations involved in Mhom fermentation in the five manufacturing sites belonged to *Lactobacilli* spp. (*L. plantarum*, *L. curvatus*, *L. delbrueckii*, *L. acidophilus*, *L. paracasei*, *L. brevis 1*, *L. brevis 3*, *L. pentosus* and *L. mesenteroides*), followed by *Pediococcus pentosaceus 1*, *Pediococcus pentosaceus 2* and *Carnobacterium divergens*, respectively. The percentage of isolation for these species was different in each of Mhom manufacturing sites. The homofermentative lactobacilli accounted for 88.73% of the isolates obtained from MRS agar.

2. The geographic distribution of *Lactobacillus* spp. in Mhom samples among the five manufacturing sites was evident. *Lactobacilli* species are characterized by manufacturing site-specific strains.

3. Molecular tools used in this study led to the understanding that some isolated LAB strains were manufacturing site-specific, whereas others shared a degree of homology independ of the source of fermentation. The results highlighted the capability of isolated LAB strains to adapt to specific and local fermentation conditions.

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

Raw materials and ingredients, such as unpeeled and peeled garlic, beef, liver and spleen, were evaluated for their microbiological load, including aerobic plate count, and counts of LAB, *Micrococcaceae*, coliforms, enterococci, *Escherichia coli* O157: H7, *Staphylococcus aureus*, yeasts and molds. The results were summarized as follows:

1. The initial micro flora found in Mhom samples originated mainly from raw materials and ingredients used in the manufacturing process.

2. The highest total viable count was found in the natural casings.

3. Yeasts and molds were mainly found in beef, liver and spleen.

4. The high LAB counts were observed in spleen, natural casings, beef and liver.

5. *Micrococcaceae* counts remain relatively high in the liver, spleen, natural casings and beef.

6. The numbers of enterococci were found mainly in the spleen, beef, liver and natural casings.

7. *Staphylococcus aureus* and coliforms counts were detected in spleen, natural casings, beef and liver, with their highest counts found in beef.

8. *E. coli* O157:H7 was not detected in any of unpeeled garlic and peeled garlic, as well as beef, liver, spleen and natural casings.

Several LAB strains were isolated from Mhom using MRS agar based on their gram-positive staining, and indole-, catalase-, motility- and spore forming-negative, and rod-shaped. LAB isolates were firstly screened for their antimicrobial activity using the modified disc diffusion method. The antimicrobial activity of LAB isolates was tested in duplicate against the indicator bacteria, including *Staphylococcus aureus* strains ATCC13565, ATCC25923 and ATCC25904, *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhi*, *Enterococcus faecalis*, *Pseudomonas* sp. and *Staphylococcus epidermidis*. The results were summarized as follows:

1. LAB isolates exhibited *in vitro* inhibitory activity against most of the bacteria indicator strains tested in this study. This indicates that with the use of the agar disc diffusion method the inhibitory compounds produced by the LAB isolates were extracellular and diffusible because the inhibition took place by diffusing through a layer of agar.

2. Of all indicator bacteria used in this study, *Ent. faecalis*, *S. typhi*, *E. coli* O157:H7, *S. aureus* strain ATCC25923, *B. cereus* and *L. monocytogenes* were the most sensitive pathogenic microbes to all LAB isolates, whereas *S. epidermidis*, *S. aureus* ATCC13565 and *Pseudomonas* sp. revealed weak positive results. All LAB isolates exhibited strong antimicrobial activities when tested against *Enterococcus faecalis* and *S. typhi*.

3. HPLC-UV analysis revealed that the amounts of lactic acid produced in the cell-free supernatants of most of the LAB isolates were higher than 5 g/l.

4. As our LAB were grown in MRS broth media containing glucose as the primary carbon source, the observed inhibition might arise from the acid produced. When the pH values of the culture supernatants were adjusted to 6.5 with NaOH, there was significant reduction of inhibition activity against some pathogenic bacteria.

Taken together, our results indicated that the inhibitory substance may be due to organic acids and/or acid derivatives and/or bacteriocin produced and released by LAB. Any of LAB strains with high osmotolerance would be desirable as an industrial potential.

Five LAB isolates, such as CP116, CP120, CP210, MK114 and KS103, were finally characterized in the primary screening based on their high antimicrobial properties, as well as lactic acid productivity, and were further evaluated utilizing the secondary screening approach to determine their growth rate, acidifying capacity and tolerance to the extreme environmental conditions, such as high temperatures, high concentrations of lactic acid and sodium chloride, and low pH. The results were summarized as follows:

1. Most of the LAB isolates could grow at a wide range of temperature ranging from 15 to 45°C, while the CP116 could not grow at 15°C. The ability of LAB to grow at high temperature (30–45 °C) is a desirable trait as it could translate to increased rate of growth and lactic acid production. At the same time, a high fermentation temperature reduces contamination by other microorganisms.

2. Three LAB isolates from CP1 (CP120), CP2 (CP210) and MK (MK114), were the most tolerant to high lactic acid concentrations as they were able to grow at 7.5%, whereas KS 103 and CP116, were the most sensitive to low lactic acid concentrations as its growth was indicated only at 2.5%. None of the five isolates grew at 10% and 15% lactic acid concentrations. A higher tolerance to high lactic acid concentrations is a desirable trait for an industrial strain of LAB as it could produce more lactic acid in the fermentation broth without prematurely affecting itself adversely.

3. Similar to their high tolerance to lactic acid concentrations, CP120, CP210 and MK114 isolates were the most tolerant to high NaCl concentration, whereas the other isolates did not possess the ability to grow at the highest salt concentration, i.e., 10%. These findings provided an indication of the osmotolerance level of the LAB strains tested in this study.

4. All LAB isolates could grow at pH 4.5 and pH 7.0., but could not grow in an alkaline environment, pH 9, indicating that the LAB isolates tested in this study was most likely to be the acidophilic bacteria.

5. The growth characteristic and lactic acid production profiles of the selected CP120, CP210 and MK114 isolates, based on their highest tolerance to the extreme environmental conditions, were investigated in the time-course study to generate their growth and lactic acid production profiles. These results demonstrated that CP210 had high efficacy in converting glucose to lactic acid. CP210 and CP120 had similar tolerance levels to high temperature (up to 45°C), lactic acid concentration (7.5%), NaCl concentration (10%), and to low pH (4.5), but the faster production of lactic acid by CP210 might give it a slight advantage over CP120, and MK114.

L. plantarum strains CP120 and MK114 were previously isolated and selected from indigenous fermented Mhom based on their high antimicrobial activity and tolerance to the extreme environmental conditions, and then were assigned as starter cultures and different combinations were applied to the raw materials and ingredients, and let to ferment Mhom products. The microbial and physic-chemical profiles were followed and determined for dynamic changes during fermentation process. The results were summarized as follows:

1. With the use of different combinations of LAB starter cultures, the obtained Mhom products had pH values ranging from 4.62 to 4.66 at the end of the fermentation process. The drop in pH was attributed to the involvement of LAB that were able to ferment glucose to produce lactic acid, which then resulted in the lowering of the pH.

2. The percentage of weight loss of Mhom samples increased from initial values of 10.18–15.48 (day 1) to 54.36–58.33% during the ripening process. When fermentation was prolonged to 28 days, CP210 exhibited the highest weight loss. On the basis of final weight loss, in this study Mhom can be classified as the semi-dry, acid fermented sausage.

3. Glucose consumption was mainly detected during the stationary phase at pH 4.5–5.9. At the beginning of fermentation, glucose consumption was initiated earlier and maintained during the stationary phase. Maximum and minimum glucose

consumption was correlated with microbial growth but the amount of glucose produced varied according to the inoculated batches.

4. The concentrations of lactic acid increased gradually up to day 13. The combination of strains CP120+CP210 resulted in the highest amount of lactic acid ($p \leq 0.05$). Acetic acid represented a small amount of the total acids produced by all strains. For the strains CP210+MK114, acetic acid represented about half of the total acids produced during growth for 14 days. The production of acetic acid exhibited the lower amounts in the case of strain combination than the single strain ($p \leq 0.05$).

5. The population of LAB exceeded, with the exception of MK114. After 3 days of incubation, the number of LAB counts in all inoculated batches increased corresponding to the decreases in pH. After 9 days of incubation, the number of LAB counts continued to decrease.

6. Total aerobic plate counts showed the same trend as the LAB counts. Thus, fermentation of Mhom involving successive growth of different groups of microorganisms was dominated by LAB. A significant increase in total aerobic counts occurred during the first 9 days and then decreased gradually thereafter in all the inoculated batches.

7. The addition of different combinations of strains to batches had no effect on the *Micrococcaceae* counts, and their counts showed the same trend as the total aerobic plate count. The *Micrococcaceae* counts started to decrease in fermented meat after 9 days of ripening. The maximum *Micrococcaceae* counts were determined at day 7 to 9 of ripening in all the inoculated batches.

8. Initial counts of enterococci gradually decreased during the ripening period, and this phenomenon was evident in the single strain batches rather than the combination strains batches. The decrease in numbers of enterococci was a typical decrease due to environmental conditions which could make it difficult for enterococci to grow.

9. Yeast and mold counts showed the same trend with total aerobic plate counts. The increase of yeast and mold counts occurred during the first 7 days and then decreased gradually thereafter in all the inoculated batches.

10. The initial counts of *S. aureus* gradually decreased during ripening and this phenomenon was evident in the single strain batches rather than the combination

strains batches. The numbers of enterococci decreased, possibly due to the decrease in pH and oxygen limitation.

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

In the present study, *L. plantarum* was used as a starter culture for Mhom processing and compared to the controls (no inoculated culture added). The starter cultures were isolated and selected from commercial Mhom for their high antimicrobial activity and tolerance to adverse environmental conditions. Several multi-combinations of the appropriate starter cultures 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.

1. As the fermentation proceeded, Mhom inoculated with *L. plantarum* CP120 + *L. plantarum* CP210 had a greater weight loss than the controls.

2. The pH values of Mhom products generally decreased with increasing fermentation time. A rapid decrease in pH during the first 7 days was probably due to an increased amount of organic acids, mainly lactic acid produced by LAB.

3. From day 14, a slight increase in lactic acid content was observed in all treatments. This may be due to the consumption by microbial groups that are present, and to the formation of low molecular weight nitrogen compounds, i.e., ammonia and biogenic amines. However, at the end of fermentation (pH~4.6), Mhom inoculated with starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210) had lower acetic acid than the control.

4. The highest numbers of total aerobic plate counts of the controls and inoculated batches occurred at day 9 and day 5.

5. *Micrococcaceae* profiles showed significant differences ($p \leq 0.05$) between the control and the inoculated batch, which reflected the starter culture growth. The highest numbers of *Micrococcaceae* count of the control and inoculated batch observed at days 7 and 9, respectively, then gradually decreased until the end of the ripening process. At the end of the fermentation, the numbers of *Micrococcaceae* in the inoculated batch were higher than those of the control.

6. Enterococci decreased steadily in numbers both in the control and inoculated batches.

7. Starter culture had an effect on the *S. aureus*. The highest average number of *S. aureus* was determined in the controls and this value was statistically different from that of the inoculated batches ($p \leq 0.05$). The duration of the ripening period also had a significant effect on *S. aureus* count.

8. Yeasts and molds were detected during the fermentation period.

9. The results of organoleptic analysis showed similar results obtained between Mhom products fermented with *L. plantarum* CP120 + *L. plantarum* CP210 as starter culture and those fermented without the starter culture, i.e., control. However, The Mhom fermentation with the LAB starter resulted in a less sweet, musty flavor and more acidic taste compared to an un-inoculated one. These properties were highly appreciated by the sensory panel member.