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APPENDICES

APPENDIX A
BACTERIAL MEDIA

BACTERIAL MEDIA

1. LB agar

Per liter:

Tryptone	10.0	g
Yeast extract	5.0	g
Sodium chloride	10.0	g
Agar	15.0	g

Suspend 40 g of the powder (dehydrated product of Difco, USA) in 1 liter of purified H_2O. Mix thoroughly and then heat the agar medium with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 7.0 ± 0.2 at 25°C.

2. LB broth

Consists of the same ingredients as the LB agar without the agar. Suspend 25 g of the purified H_2O. Mix thoroughly and warm gently until solution is complete. Sterilize by autoclaving at 121°C for 15 minutes.

3. MRS agar

Per liter:

Peptone from casein	10.0	g
Meat extract	8.0	g
Yeast extract	4.0	g
D(+)-glucose	20.0	g
Tween 80	1.0	g
Diammonium hydrogen citrate	2.0	g
Sodium acetate	5.0	g
Magnesium sulfate	0.2	g
Manganese sulfate	0.04	g
Agar	15.0	g

Dissolve 62.2 g of the powder (dehydrated product of Merck, Germany) in 1 liter of purified H_2O. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 5.7 ± 0.2 at 25°C.

4. MRS broth

Consists of the same ingredients as the MRS agar without the agar. Dissolve 52.2 g of the powder (dehydrated product of Merck, Germany) in 1 liter of purified H_2O. Mix thoroughly and warm gently until solution is complete. Sterilize by autoclaving at 121°C for 15 minutes.

5. BHI (Brain heart infusion) agar

Per liter:

Calf Brain, Infusion from 200 g	7.7	g
Beef Heart, Infusion from 200 g	9.8	g
Proteose peptone	10.0	g
Dextrose	2.0	g
Sodium chloride	5.0	g
Disodium phosphate	2.5	g
Agar	15.0	g

Suspend 52 g of the powder (dehydrated product of Difco, USA) in 1 liter of purified H_2O. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 7.4 ± 0.2 at 25°C.

6. BHI (Brain heart infusion) broth

Consists of the same ingredients as the BHI (Brain heart infusion) agar without the agar. Dissolve 37 g of the powder (dehydrated product of Difco, USA) in 1 liter of purified H_2O. Mix thoroughly and warm gently until solution is complete. Sterilize by autoclaving at 121°C for 15 minutes.

7. Mannitol salt agar (MSA)

Mannitol salt agar or MSA is a commonly used growth medium in microbiology. It contains a high concentration (~7.5%-10%) of salt (NaCl), making it selective for Staphylococci (and *Micrococcaceae*) since this level of NaCl is inhibitory to most other bacteria. It is also a differential medium, containing mannitol and the indicator phenol red. Coagulase-positive *Staphylococci* produce yellow colonies with yellow zones, whereas coagulase-negative Staphylococci produce small pink or red colonies with no color change to the medium. It is used for the selective isolation of presumptive pathogen (pp) Staphylococci.

Per liter:

Enzymatic digest of casein	5.0	g
Enzymatic digest of animal tissue	5.0	g
Beef extract	1.0	g
D-mannitol	10.0	g
Sodium chloride	75.0	g
Phenol red	0.025	g
Agar	15.0	g

Suspend 111.025 g of the powder (dehydrated product of Difco, USA) in 1 liter of purified H_2O. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 7.4 ± 0.2 at 25°C.

8. Plate Count Agar

Per liter:

Dextrose	1.0	g
Tryptone (vegetable)	5.0	g
Yeast extract	2.5	g
Agar	15.0	g

Suspend 23.50 g of the powder in 1 liter of purified water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 7.0 ± 0.2 at 25°C.

9. MacConkey agar

MacConkey agar (or McConkey agar) is a culture medium designed to grow Gram-negative bacteria and stain them for lactose fermentation. It contains bile salts (to inhibit most Gram-positive bacteria, except *Enterococcus* and some species of *Staphylococcus* i.e. *Staphylococcus aureus*), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which stains microbes fermenting lactose), lactose and peptone. A variant, Sorbitol-MacConkey agar, (with the addition of additional selective agents) can assist in the isolation and differentiation of enteropathogenic *E. coli* serotypes such as *E. coli* O157:H7, by the presence of white circular colonies that are non-lactose fermenting.

Per liter:

Peptone 190 (pancreatic digest of gelatin)	17.0	g
Peptone 180 (Animal tissue - casein polypeptone)	3.0	g
Lactose	10.0	g
Bile salt	31.5	g
Sodium choride	5.0	g
Agar	5.0	g
Nuetral red	0.03	g
Crystral violet	1.001	g

Suspend 72.531 g of the powder in 1 liter of purified water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 7.1 ± 0.2 at 25°C.

10. SF agar

For diagnostic microbiology purposes, the medium is useful in differentiation of enterococci from streptococci. Pure cultures of streptococci are inoculated into SF Medium in order to determine if the respective culture is *Enterococcus* sp. Enterococci ferment dextrose and grow in the presence of the inhibitor sodium azide. Peptone and dextrose supply the nutrients required for the growth of enterococci. Sodium chloride maintains the osmotic balance of the medium. Sodium azide exhibits a bacteriostatic effect on gram-negative bacteria through its inhibitory action on enzymes in the electron transport system. Bromcresol purple serves as a pH indicator.

Per Liter

Tryptone	20.0	g
Dextrose	5.0	g
Dipotassium Phosphate	4.0	g
Monopotassium Phosphate	1.5	g
Sodium Chloride	5.0	g
Sodium Azide	0.5	g
Bromcresol Purple	32.0	mg

Dissolve 36 g of the powder in 1 liter of purified water. For double strength medium, use 72 g/l of purified water. Rehydrate with proportionally less water when liquid inocula will exceed 1 ml. Autoclave at 121°C for 15 minutes. Test samples of the finished product for performance using stable, typical control cultures. Inoculate tubes of the medium with pure cultures of the test organisms. Incubate tubes for 18-48 hours at 45-46°C in an aerobic atmosphere.

A positive reaction is indicated by turbidity and a yellow-brown color due to the fermentation of dextrose and the resultant color change of the bromcresol purple indicator. A negative reaction is indicated by no change in the purple color of the medium.

Streptococci yielding positive reactions:

E. faecalis

E. faecium

Streptococci yielding negative reactions:

S. bovis

S. equinus

S. mitis

S. salivarius

11. Baird-Parker medium with egg yolk tellurite emulsion

A selective medium for the isolation of *staphylococci* from food samples. Sodium pyruvate protects damaged cells and aids their recovery. Egg yolk emulsion assists in the diagnosis of organisms. Glycine, lithium and tellurite suppress the growth of other bacteria in foods without inhibiting *Staphylococcus aureus*. *S. aureus* reduces tellurite to form grey-black shiny colonies and produces clear zones around the colonies due to the proteolytic activity on egg yolk. On further incubation most *S. aureus* strains produce opaque zones around their colonies due to lipase activity.

Per Liter:

Yeast extract	1.0	g
Sodium pyruvate	10.0	g
Meat extract	5.0	g
Lithium chloride	5.0	g
Glycine	12.0	g
Casein peptone	10.0	g
Agar	15.0	g

Suspend 58.00 g of the powder in 1 liter of purified water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. The final pH is 6.8 ± 0.2 at 37°C.

Expected Results

Organism	Colour of colony/comments
S. aureus	Grey-black, shiny colony. Surrounded by a zone of clearing.
Staphylococcus epidermidis	Not shiny black colony. Seldom produce clearing.
Staphylococcus saprophyticus	Irregular colony and may produce clearing. Wide opaque zones may be produced in 24h.
Micrococcus	Very small colony in shades of brown and black. No clearing.
Bacillus species	Dark matt brown colony with occasional clearing after 48h.
Escherichia coli	Large brown-black colony.
Proteus	Brown-black colony. No clearing.

12. Sabouraud agar

Sabouraud agar is a type of agar containing peptones. It is used to cultivate dermatophytes and other types of fungi. It was created by, and is named after, Raymond Sabouraud in 1892. Later adjusted by Emmons, its pH level was brought closer to the neutral range to support the growth of other subcultures of fungi.

Sabouraud agar typically contains:

40 g/l dextrose
10 g/l peptone
20 g/l agar
100mg/l Chloramphenicol
pH 5.6

APPENDIX B
REAGENTS AND BUFFERS

REAGENTS AND BUFFERS

1. Reagent and Buffers for Agarose Gel Electrophoresis

1.1 Tris-acetate (TAE) buffer

Stock solution (50X): (for 1000 ml) 242 g of Tris base,
57.1 ml of glacial acetic acid,
100 ml of 0.5 M EDTA (pH 8.0)

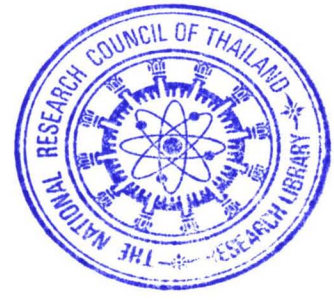
Working solution (1X): 0.004 M Tris acetate,
0.001 M EDTA.

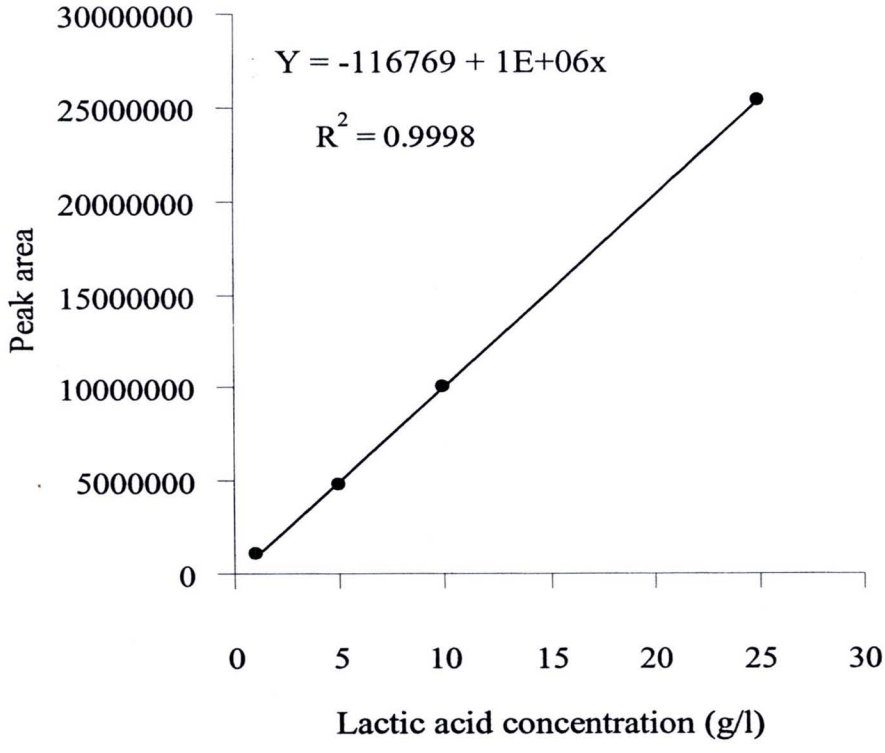
1.2 Ethidium bromide

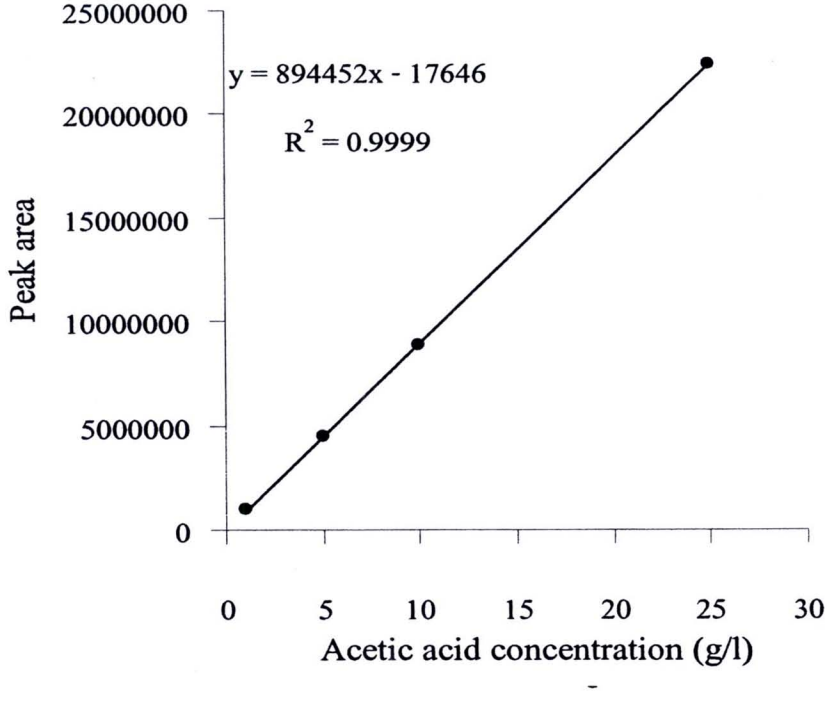
Stock solution (10 mg/ml): 1 g of ethidium bromide in 100 ml of H_2O

Working solution (0.5 μg/ml): (for 100 ml) dissolve 5 μl of ethidium bromide stock solution in H_2O to a total volume of 100 ml.

APPENDIX C
STANDARD CURVE







Standard curve of lactic acid and acetic acid

APPENDIX D
QUESTIONNAIRE FOR MHOM PANELISTS

A QUESTIONNAIRE FOR MHOM PANELISTS

Questionnair code:_____

PART 1 : Personal Information

1. Name.....

2. Age.....years old

3. Gender

☐ Male ☐ Female

4. Marital status

☐ Single ☐ Married

☐ Other

5. Occupation

☐ Secondary student ☐ Tertiary student

☐ Other (Please specified).....

.....

6. Education

☐ < High school ☐ High school

☐ 2 years of college ☐ 4 years of college/University

☐ Post-graduate studies ☐ Other (Please specified).....

.....

7. Your monthly income (or amount of money you receiced from guardian per month)

☐ ≤ $150 ☐ $ 150-300

☐ $ 300-600 ☐ >$ 600

SENSORY EVALUATION FORM

Please read each of the following questions. Then place a tick in the box that best reflects your opinions about the particular question. Please answer any sections where you are asked to give reasons for your selection or actions. With some questions you may be asked to tick more than one box. The instruction to tick more than one box will be provided after the question.

PART 2 : Mhom Behaviour

Definition

Mhom is a fermented food made from ground beef, spleen and liver. The ingredients commonly include roast rice powder, salt, sugar, herbs and spices. Typically the sausage is formed in a casing traditionally made from

Official only

1. Do you normally eat Mhom? 1.1 1 ( )

□ Yes **(Please continue with the next question)** 2 ( )

□ No **(Please state reasons)**

Why do you not consume Mhom?

Because.....

.....

.....

.....

.....

2. When was the last time you ate a Mhom?

☐ Today ☐ Yesterday

☐ Within this week ☐ Last week

☐ Two week ago ☐ Other (Please specify)

.....

.....

.....

.....

3. How often did you eat a Mhom in the last month?

☐ More than 3 times per day ☐ Three times a day

☐ Twice a day ☐ Once a day

☐ 5-6 times a week ☐ Once per two days

☐ 2-3 times a week ☐ Once a week

☐ Less than once per week ☐ Never

4. What time or times of the day do you normally eat a Mhom?

(You may tick as many boxes as you like)

☐ 7.00-9.00 am ☐ 9.00-11.00 am

☐ 11.00-1.00 pm ☐ 1.00-3.00 pm

☐ 3.00-5.00 pm ☐ 5.00-7.00 pm

☐ 7.00-9.00 pm ☐ 9.00-11.00 pm

☐ Other (Please specify).....

.....

.....

.....

Official only

1.2 1 ( ) 2 ( )
3 ( ) 4 ( )
5 ( ) 6 ( )

1.3 1 ( ) 2 ( )
3 ( ) 4 ( )
5 ( ) 6 ( )
7 ( ) 8 ( )
9 ( ) 10 ( )

1.4 1 ( ) 2 ( )
3 ( ) 4 ( )
5 ( ) 6 ( )
7 ( ) 8 ( )
9 ( )

PART 3 : Mhom Panelists

Please taste these samples and evaluate how much you like their appearance, color, uniform color, crustiness, sweetness, saltiness, acid taste, bitterness, glutamate, mustiness, hardness, cohesiveness and fat melting and also how much you like the product. A nine-point hedonic scale (1 = dislike extremely, → 9 = like extremely) is used for describing the product. Please fill a number that corresponds to how you feel about each samples.

Sample Code.....

	Attribute											
	Color	Uniform color	Crustiness	Sweetness	Saltiness	Acid taste	Bitterness	Glutamate	Mustiness	Hardness	Cohesiveness	Fat melting
9 = Like extremely	9	9	9	9	9	9	9	9	9	9	9	9
8 = Like very much	8	8	8	8	8	8	8	8	8	8	8	8
7 = Like moderately	7	7	7	7	7	7	7	7	7	7	7	7
6 = Like slightly	6	6	6	6	6	6	6	6	6	6	6	6
5 = Neither like or dislike	5	5	5	5	5	5	5	5	5	5	5	5
4 = Dislike slightly	4	4	4	4	4	4	4	4	4	4	4	4
3 = Dislike moderately	3	3	3	3	3	3	3	3	3	3	3	3
2 = Dislike very much	2	2	2	2	2	2	2	2	2	2	2	2
1 = Dislike extremely	1	1	1	1	1	1	1	1	1	1	1	1

APPENDIX E
ANALYSIS OF VARIANCE

Table E1 Analysis of variance for chemical and microbiological analysis of Mhom

		Sum of Squares	df	Mean Square	F	Sig.
Aerobic plate count	Between Groups	2.4E + 16	4	6.0E + 15	721.356	0.000
	Within Groups	4.2E +13	5	8.4E + 12		
	Total	2.4E + 16	9			
Yeast and Mold	Between Groups	3.3E + 16	4	8.1E + 15	19994.340	0.000
	Within Groups	2.0E + 12	5	4.1E + 11		
	Total	3.3E + 16	9			
Lactic acid bacteria	Between Groups	4.0E + 13	4	9.9E + 12	24.758	0.002
	Within Groups	2.0E + 12	5	4.0E + 11		
	Total	4.2E + 13	9			
Micrococcaceae	Between Groups	8.3E + 11	4	2.1E + 11	202831.97	0.000
	Within Groups	5.1E + 06	5	1.0E + 06		
	Total	8.3E + 11	9			
Enterococci	Between Groups	4.7E + 13	4	2.0E + 12	1184269.5	0.000
	Within Groups	5.0E + 07	5	1.0E + 07		
	Total	4.7E + 13	9			
Staphylococcus aureus	Between Groups	1.1E + 13	4	2.8E + 12	284144.6	0.000
	Within Groups	5.0E + 07	5	1.0E + 07		
	Total	1.1E + 13	9			
coliforms	Between Groups	5.4E + 07	4	1.4E + 07	79418.870	0.000
	Within Groups	853.000	5	170.600		
	Total	5.4E + 07	9			
pH	Between Groups	0.463	4	0.116	236.449	0.000
	Within Groups	0.002	5	0.000		
	Total	0.466	9			
Total acidity	Between Groups	0.786	4	0.196	258.421	0.000
	Within Groups	.004	5	0.001		
	Total	.789	9			

Table E2 Analysis of variance for microbiological analysis of raw material for Mhom process

		Sum of Squares	df	Mean Square	F	Sig.
Aerobic plate count	Between Groups	4.2E + 11	4	1.0E + 11	132.629	.000
	Within Groups	7.9E + 09	10	7.9E + 08		
	Total	4.3E + 11	14			
Yeast and Mold	Between Groups	1.2E + 10	4	2.9E + 10	97.154	.000
	Within Groups	3.0E + 08	10	2.9E + 07		
	Total	1.2E + 10	14			
Lactic acid bacteria	Between Groups	2.1E + 09	4	5.3E + 09	37.909	.000
	Within Groups	1.4E + 08	10	1.4E + 08		
	Total	2.2E + 09	14			
Micrococcaceae	Between Groups	7.6E + 09	4	1.9E + 10	48.742	.000
	Within Groups	3.9E + 08	10	3.9E + 07		
	Total	8.0E + 09	14			
Enterococci	Between Groups	5.2E + 08	4	1.3E + 08	27.375	.000
	Within Groups	4.8E + 07	10	4.8E + 06		
	Total	5.7E + 08	14			
Staphylococcus aureus	Between Groups	2.0E + 11	4	5.1E + 10	217.962	.000
	Within Groups	2.3E + 09	10	2.3E + 08		
	Total	2.1E + 11	14			
coliforms	Between Groups	5.5E + 10	4	1.4E + 10	238.038	.000
	Within Groups	5.8E + 08	10	5.8E + 07		
	Total	5.6E + 10	14			

Table E3 Analysis of variance for sensory evolution results of the sausages manufactured with or without a starter culture at the end of the fermentation

		Sum of Squares	df	Mean Square	F	Sig.
Color	Between Groups	0.001	1	0.001	1.125	0.400
	Within Groups	0.002	2	0.001		
	Total	0.003	3			
Uniform color	Between Groups	0.002	1	0.002	0.301	0.638
	Within Groups	0.013	2	0.007		
	Total	0.015	3			
Crustiness	Between Groups	0.001	1	0.001	0.610	0.517
	Within Groups	0.002	2	0.001		
	Total	0.003	3			
Sweetness	Between Groups	0.384	1	0.384	192.200	0.005
	Within Groups	0.004	2	0.002		
	Total	0.388	3			
Saltiness	Between Groups	0.001	1	0.001	0.450	0.571
	Within Groups	0.004	2	0.002		
	Total	0.005	3			
Acid taste	Between Groups	1.392	1	1.392	1740.500	0.001
	Within Groups	0.002	2	0.001		
	Total	1.394	3			
Bitterness	Between Groups	0.002	1	.002	1.231	0.383
	Within Groups	0.003	2	0.001		
	Total	0.004	3			
Glutamate	Between Groups	0.002	1	0.002	0.516	0.547
	Within Groups	0.008	2	0.004		
	Total	0.010	3			
Mustiness	Between Groups	11.222	1	11.222	1156.959	0.001
	Within Groups	0.019	2	0.010		
	Total	11.242	3			
Hardness	Between Groups	0.001	1	0.001	0.410	0.588
	Within Groups	0.003	2	0.002		
	Total	0.004	3			
Cohesiveness	Between Groups	0.000	1	0.000	0.022	0.895
	Within Groups	0.002	2	0.001		
	Total	0.002	3			
Fat melting	Between Groups	0.001	1	0.001	1.059	0.412
	Within Groups	0.002	2	0.001		
	Total	0.003	3			

APPENDIX F
RESEARCH PUBLICATION

Microbiological Characteristics of "Mhom", a Thai Traditional Meat Sausage

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Abstract

A Thai traditional meat sausage, namely Mhom, is a popular fermented meat product in the northeastern region of Thailand and yet little scientific information about Mhom is known. Seventy- one isolates of lactic acid bacteria were isolated from Mhom made in four provinces i.e. Chaiyaphum (CP), Khonkaen (KK), Maha Sarakham (MK), and Kalasin (KS). It was found that the pHs of Mhom coded CP1, CP2, KK, MK and KS were 4.74, 4.94, 4.77, 4.30 and 4.76, respectively, and % total acidity as lactic acid were 1.16, 0.78, 1.56, 0.94, and 0.86, respectively. In addition, the total lactic acid bacteria in the products were 6.0×10^6, 7.8×10^6, 5.4×10^6, 6.4×10^6 and 1.0×10^7 cfu/g for Mhom coded CP1, CP2, KK, MK and KS, respectively. The isolates were then subjected to biochemical tests using the API 50 CH and they were identified as *Lactobacillus curvatus, L. delbrueckii, L. acidophilus, L. paracasei, L. brevis, L. pentosus, L. mesenteroides, L. plantarum, L. farciminis, Carnobacterium divergens, Pediococcus pentosaceus* and *Enterococcus.* These strains of lactic acid bacteria found in Mhom may play significant roles in the ripening process resulting in unique sensory characteristics in the end products. They were naturally selected from environmental microbiota by the ability to best compete under the prevailing conditions of the ecological niche. The lactic acid bacteria isolated and identified could be used as starters in Mhom production to obtain a more uniform sensory quality in the end products without undesirable flavors and microorganisms that may often be found in a natural fermentation in Mhom and the products could also be consumed safely.

Key words: Lactic acid bacteria; Mhom; fermented meat product

1. Introduction

Lactic acid bacteria (LAB) and *Micrococcaceae* strains are important microorganisms used as starter cultures in meat fermentations. Their addition to meats may improve product safety and stability, provide diverse sensory characteristics and health benefits from probiotic characteristics as reported in a recent review by Lucke (2000). The most frequently isolated lactic acid bacteria from dry sausages processed with different technologies are *Lactobacillus sakei*, *L. curvatus* and *L. plantarum* (Papamanoli et al., 2003; Comi et al., 2005). The isolation and selection of lactic acid bacteria as starter cultures for meat fermentation presents a considerable challenge to standardization and management of quality of dry fermented sausage.

With the different traditional formulations of dry-fermented sausages, physico-chemical modifications occur including dehydration, fermentation of carbohydrates and acidification, development of a typical colour, lipolysis and oxidation of lipids, and proteolysis (Ordonez et al., 1999). These changes are responsible for the organoleptic characteristics of the final products (Ordonez et al., 1999).

Mhom is a Thai traditional dry fermented sausage made from meats including pork or beef as a principal component along with internal organs i.e. liver or spleen and other ingredients such as salt, fried or cooked rice, garlic, and pepper. The raw material and ingredients are mixed, casing stuffed and tied. Then they are kept about 2-3 days for fermentation. Mhom could be consumed raw or cooked. This fermented meat product has been widely developed and consumed in the northeastern region of Thailand. The fermentation is naturally mediated by indigenous bacteria resulting in rather non-uniform final products (Thiravattanamontri et al., 1998). The bacteria found most commonly in Thai fermented meat products are lactobacilli, pediococci and micrococci. The precise roles of these bacteria on the quality of the products have not been fully understood (Thiravattanamontri et al., 1998). Developments in meat fermentation have been focusing on application of defined starter cultures for a better control of fermentation and product consistency (Kastnera et al., 2006). In addition to difficulties in obtaining product consistency, pathogens in raw fermented sausage still cause food safety problems.

The study of the ecology of fermented sausages is of primary importance to understand the physical and chemical changes during fermentation and maturation. The objectives of this study were to determine the different microbial groups in Thai traditional fermented meat sausage (Mhom) and identify the microorganisms involved.

2. Materials and methods

2.1 Samples

The experiment was carried out at the Department of Food Technology, Khon Kaen University, Khon Kaen, Thailand. Five Mhom samples were collected from the local markets in the Northeastern region of Thailand including Khon Kaen, Maha Sarakham, Chaiyaphum and Kalasin provinces. Mhom were produced according to the traditional method (without the use of starter cultures). The basic initial sausage mixture included beef meat, liver, salt, fried or cooked rice, pepper, sucrose, garlic, and spices. Samples were taken immediately after casing, 30 days after ripening and then transported to the laboratory under refrigeration (below 4 °C).

2.2 Microbiological analysis

Mhom samples were assayed for (1) aerobic plate counts (2) lactic acid bacteria (3) Micrococcaceae (4) Coliforms (5) Enterococci (6) *Escherichia coli* O157:H7 (7) *Staphylococcus aureus*, and (8) Yeasts and Molds. Ten grams of each sample were aseptically transferred to a sterile stomacher bag with 90 ml of saline-peptone water (NaCl, 2%; bacteriological peptone, 0.1% and Tween 80, 1%). The preparation was mixed for 3 min in a stomacher. Additional decimal dilutions were prepared, and the following analyses were carried out: (1) aerobic plate counts on Plate Count agar incubated for 48 h at 30 °C (2) lactic acid bacteria on MRS agar incubated for 48 h at 30 °C under restricted oxygen conditions in an anaerobic jar (3) Micrococcaceae on mannitol salt agar (MSA) incubated for 48 h at 30 °C (4) Coliforms on MacConkey agar incubated for 24 h at 37 °C (5) Enterococci on *Streptococcus faecalis* (SF) agar incubated for 72 h at 42 °C (6) *Escherichia coli* O157:H7 on Sorbitol MacConkey Agar (SMAC) incubated for 24 h at 37 °C (7) *Staphylococcus aureus* on Baird-Parker medium with egg yolk tellurite emulsion

incubated at 37 °C for 24 to 48 h and (8) Yeast and Molds on Sabouraud agar incubated for 48 to 72 h at 30 °C. After counting, means and standard deviations were calculated. Ten colonies from MRS plates for each sample were randomly selected, transferred to MRS and brain hearth infusion (BHI) broth, incubated overnight at appropriate temperatures and stored at –80 °C in the same liquid media containing 30% glycerol for further study. All isolates were preliminarily characterized for cell morphology and Gram's reaction.

2.3 Characterization and identification of microbial isolates from Mhom samples

Pure culture colonies from MRS plates were tested for Gram's reactions. Gram-positive isolates were stored at – 80°C in MRS broth with 30% glycerol (v/v) (Merck, Darmstadt, Germany). The isolates were activated by two consecutive transfers in broth before use and identified to species level according to the methodology and characteristics described by Curk, Hubert, & Bringel (1996). The carbohydrate fermentation profiles of the isolates were investigated using API 50 CH strips and API CHL medium according to manufacturer's instructions (API system, Bio-Merieux, France) (Ammor *et al.*, 2005).

2.4 Chemical analysis

Total acidity as lactic acid and pH were determined using the methods of Franco et al. (2002). All chemical determinations were made in triplicate for each sample.

2.5 Statistical analysis

All the data reported below were evaluated in triplicate, in each of the samples. The statistical analysis of the data was carried out by analysis of the variance (ANOVA) and the Duncan's new multiple range test to show measurements which can be considered statistically different. A significance level of $\alpha = 0.05$ was used.

3. Results

3.1 Counts of the different microbial groups and their relationships with chemical properties

Table 1 shows the chemical properties and the counts of the different microbial groups of Mhom samples collected from Chaiyaphum (CP), Khon Kaen (KK), Maha Sarakham (MK), and Kalasin (KS) provinces. In general, the pH values of ready-to-eat Mhom samples (20–30 days of fermentation/ripening) were virtually similar, with the values in the range of 4.30 to 4.94 as the pH values of the samples coded CP1, CP2, KK, MK and KS were 4.74, 4.94, 4.77, 4.30 and 4.76 respectively, with the average value of 4.70. The MK coded sample was found to have the lowest pH value (4.30). Moreover, the % total acidities as lactic acid were 1.16, 0.78, 1.56, 0.94 and 0.86 for samples coded CP1, CP2, KK, MK and KS, respectively, with the average value of 1.06. The highest acidity ($p \leq 0.05$) was observed in the KK coded sample.

Table 1 Chemical and microbiological analysis of ripened Mhom samples ($n = 5$).

Microorganism (cfu/g)	Sources					
	CP1	CP2	MK	KK	KS	Average
pH	4.74b	4.94a	4.30c	4.77b	4.76b	4.70
Total acidity (%)	1.16b	0.78d	0.94c	1.56a	0.86cd	1.06
Aerobic plate count	6.0×10^7 b	6.1×10^6 d	1.4×10^8 a	2.0×10^7 c	3.2×10^7 c	5.0×10^7
Yeast and Mold	7.3×10^6 e	3.9×10^7 b	1.2×10^7 d	2.6×10^7 c	1.6×10^8 a	4.9×10^7
Lactic Acid Bacteria	6.0×10^6 b	7.8×10^6 b	5.4×10^6 b	6.4×10^6 b	1.0×10^7 a	7.1×10^6
Micrococcaceae	2.7×10^4 d	7.5×10^5 a	2.8×10^4 d	3.7×10^4 c	3.9×10^5 b	2.5×10^5
Enterococci	4.6×10^4 d	6.8×10^4 c	1.9×10^4 e	9.6×10^4 b	5.5×10^5 a	1.6×10^5
Staphylococcus aureus	3.3×10^3 d	2.7×10^6 a	9.8×10^3 d	6.3×10^4 c	8.5×10^4 b	5.7×10^5
Coliforms	2.4×10^2 d	9.6×10^2 b	2.2×10^2 d	6.2×10^3 a	3.3×10^2 c	1.6×10^3
E. coli 0157:H7	ND	ND	ND	ND	ND	

LAB: Lactic acid bacteria, CP: Chaiyaphum, MK: Maha Sarakham, KK: Khon Kaen, KS: Kalasin.

Total acidity (% acidity as lactic acid), ND : not detected.

Superscript letters with different letters in the same row, indicate significant difference ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

The results of the microbiological analyses of ripened Mhom are shown in Table 1. The Mhom samples showed high counts of all microbial groups indicating high microbial contamination in the raw materials used in their manufacture. Mhom from the five sources showed a total aerobic count in the range of 6.1×10^6 to 1.4×10^8 cfu/g and MK coded sample was found to have the highest aerobic plate count (1.4×10^8 cfu/g) while CP2 coded sample was found to have the

lowest count (6.1 x 10^6 cfu/g). Also, the yeast and mold counts were found in the range of 7.3x10^6 to 1.6x10^8 cfu/g with the average of 4.9x10^7 cfu/g. In addition, LAB counts were 6.0 x 10^6, 7.8 x 10^6, 5.4 x 10^6 and 6.4 x 10^6 cfu/g for samples coded CP1, CP2, MK and KK, respectively which were not significantly different. Furthermore, *Micrococcaceae* counts were found in the range of 2.70 x 10^4 to 7.50 x 10^5 cfu/g with the average of 2.50 x 10^5 cfu/g. The enterococci counts of Mhom samples from different locations were statistically significant. The highest enterococci count was found in KS coded sample. The *Staphylococcus aureus* counts also varied in the range of 3.30 x 10^3 to 2.70 x 10^6 cfu/g. The numbers of *S. aureus* in CP2 coded sample was the highest whereas the one in CP1 coded sample was the lowest ($p \leq 0.05$). Coliform counts were found lower than 10^4 cfu/g in all samples. *E. coli* O157:H7 was not detected in all samples.

3.2 Microbial isolation and identification

Table 2 and 3 show strain identities of the isolates from Mhom samples by using the BioMérieux (France) API-50 CH rapid test and distribution of those ones in Mhom samples among five different sources in Northeastern region of Thailand.

The results revealed the identities of the 71 isolates, with isolates from MRS agar identified as 63 lactobacilli including *Lactobacillus plantarum* (44 isolates), *Lactobacillus delbrueckii* (4 isolates), *Lactobacillus paracasei* (4 isolates), *Lactobacillus curvatus* (3 isolates), *Lactobacillus acidophilus* (2 isolates), *Lactobacillus brevis 1* (2 isolates), *Lactobacillus brevis 3* (2 isolates), *Lactobacillus pentosus* (1 isolate), and *Lactobacillus mesenteroides* (1 isolate) with 88.73% of the extensively dominant isolates grown on MRS agar along with *Pediococcus pentosaceus 1* (5 isolates), *Pediococcus pentosaceus 2* (2 isolates) and *Carnobacterium divergens* (1 isolate). Obviously, *Lactobacillus plantarum* was the most frequently isolated species (61.97%) from Mhom. Also, *L. delbrueckii* presented a similar proportion to that of *L. paracasei*. The distributions of the strains isolated from the 5 sources of Mhom samples are shown in Table 3. *L. plantarum* was the most frequently isolated species (61.97% of the total isolates) and has been shown to predominate in Mhom products. Similar percentages of strains isolated from Mhom were *Pediococcus pentosaceus 1, L. delbrueckii, L. paracasei* and *L. curvatus* as 7.04,

5.63, 5.63 and 4.23%, respectively. The largest number of isolates identified as LAB was found in CP1 (43.66% of the total isolates), followed by MK, CP2, KK, and KS, respectively.

Table 2 LAB strains isolated from Mhom.

Strain number	API-50 CH identification	ID (%)
CP1–1, CP1–2, CP1–5, CP1–6, CP1–7, CP1–8, CP1–9, CP1–10, CP1–11, CP1–12, CP1–13, CP1–15, CP1–16, CP1–17, CP1–18, CP1–20, CP1–21, CP1–22, CP1–24, CP1–25, CP1–27, CP1–29, CP2–1, CP2–2, CP2–3, CP2–4, CP2–5, CP2–7, CP2–8, CP2–11, CP2–14, MK–1, MK–4, MK–5, MK–6, MK–7, MK–8, MK–10, MK–11, MK–13, MK–14, KK–2, KK–5, KS–14	*Lactobacillus plantarum*	99.9
CP1–3, KK–3, KK–4	*Lactobacillus curvatus*	99.7
CP1–4, KK–1, MK–2, MK–12	*Lactobacillus delbrueckii*	97.3
CP1–14, CP1–23	*Lactobacillus acidophilus*	99.0
CP1–26, CP1–28, CP2–6, MK–19	*Lactobacillus paracasei*	98.3
CP1–19, CP1–31	*Lactobacillus brevis 1*	87.3
CP2–9, CP2–13	*Lactobacillus brevis 3*	99.5
KK–14	*Lactobacillus pentosus*	98.0
CP1–30	*Lactobacillus Mesenteroides*	96.9
MK–3	*Carnobacterium divergens*	99.5
CP2–12, MK–20, KS–1, KS–5, KS–15	*Pediococcus pentosaceus 1*	99.9
KS–3, KS–13	*Pediococcus pentosaceus 2*	99.9

Table 3 Distribution of LABs in Mhom from five different sources in Northeastern region of Thailand. Identification based on API 50 CH.

Species	Numbers of isolates identified						
	CP1	CP2	MK	KK	KS	Total isolates	(%)
Lactobacillus plantarum	22	9	10	2	1	44	61.97
Lactobacillus curvatus	1	-	-	2	-	3	4.23
Lactobacillus delbrueckii	1	-	2	1	-	4	5.63
Lactobacillus acidophilus	2	-	-	-	-	2	2.82
Lactobacillus paracasei	2	1	1	-	-	4	5.63
Lactobacillus brevis 1	2	-	-	-	-	2	2.82
Lactobacillus brevis 3	-	2	-	-	-	2	2.82
Lactobacillus pentosus	-	-	-	1	-	1	1.41
Lactobacillus Mesenteroides	1	-	-	-	-	1	1.41
Carnobacterium divergens	-	-	1	-	-	1	1.41
Pediococcus pentosaceus 1	-	1	1	-	3	5	7.04
Pediococcus pentosaceus 2	-	-	-	-	2	2	2.82
Total isolates	31	13	15	6	6	71	100
(%)	43.66	18.31	21.13	8.45	8.45	100	

Discussions

Mhom, a Thai traditional sausage, is characterized by acidity with final pH of about 4.5. The samples in this study were collected from 4 different locations in the Northeastern region of Thailand i.e., Chaiyaphum (CP), Khon Kaen (KK), Maha Sarakham (MK), and Kalasin (KS) provinces. The average pH value (4.7) of the Mhom after ripening was suitable for consumption which was lower than the pH values of some fermented meat products reported in the literature reviews (Comi et al., 2005; Fontana, Cocconcelli, & Vignolo, 2005). However, the average pH value of the Mhom was similar to those obtained by other researchers in various sausages (Salgado et al., 2005; Visessanguan et al., 2005). The low pH greatly extends its shelf life by inhibiting the growth of many food spoilage and pathogenic bacteria. Since it is normally uncooked, to ascertain the safety of sausages from survival and growth of some pathogenic microorganisms naturally contaminated in meat, it is recommended that sausages with pH lower than 4.6 are safe for consumption (Paukatong, & Kunawasen, 2001; Valyasevi, & Rolle. 2002). The low pH caused by the high acidifying capability of the indigenous lactic acid bacteria microflora results in the reduction of *Micrococcaceae* and Enterococci counts (Raccah, 1992). As expected, the values of total acidity had an inverse relationship to pH in the products. This was evident in CP2 samples with an average total acidity value of 0.78%, which had a correspondingly higher pH (4.94) than other samples. In general, the total acidity increased significantly in the first seven days of ripening and, afterwards, more slowly until the 14th day of ripening, when it gradually decreased until the end of the ripening process. The decrease of the values of total acidity could be related to the development of microbial flora, fundamentally moulds and yeasts, capable of consuming lactic acid (Salgado et al., 2005).

During the last few years novel benefits of adding specific LAB strains to our food has been discovered. LAB play an important role in the ripening of fermented meat, improve safety and stability of the sausages and provide health benefits by probiotic characteristics as reported in a recent review by Lucke (2000). In agreement with the results obtained by other authors in previous studies carried out on other meat sausages (Gonzalez, & Diez, 2002), the LAB were the major microbial groups. The average counts of LAB observed in our study was 7.10×10^6 cfu/g. This average value was similar to those observed in androlla, a Spanish traditional pork sausage

(Fontan et al., 2007), Botillo, a Spanish traditional pork sausage (Maria et al., 2007) and Greek fermented sausage (Drosinos et al., 2005) and they are slightly higher than those observed in sausages produced in the North East of Italy (Comi et al., 2005), dry-cured bacon, a Spanish traditional meat product (Vilar et al., 2000) and buffalo sausage (Sachindra et al., 2005).

Fermented sausages are considered good substrates for the growth of moulds and yeasts. Studies carried out with different yeast starters (mainly *Debaryomyces hansenii*, *Candida famata*) have shown the contribution of yeasts to the development of colour (by removing the oxygen) and flavour (Juan-Pablo et al., 2000), due to their ability to degrade peroxides, lipolytic activity and, to a lesser degree, proteolytic activity. Furthermore, yeasts protect sausages from the adverse effects of light. The counts of moulds and yeasts were in the range of 7.30×10^6 cfu/g to 1.60×10^8 cfu/g. This may indicate the differences in sanitation practices of different manufacturing houses. Mould and yeast counts found in this study were quite similar to those reported in sucuk, a Turkish dry-fermented sausage (Aksu, & Kaya, 2004). However, average mould and yeast counts in Mhom were slightly higher than those reported in androlla, a Spanish traditional pork sausage (Fontan et al., 2007) and sausages produced in the North East of Italy (Comi et al., 2005). Mould and yeast contamination in fermented meat products is common due to the microbial contamination of raw materials used in their manufacture, poor handling of sausage during processing and storage. However, these moulds and yeasts are not all pathogenic. In addition, the yeast counts help to explain the lactic acid and pH level in fermented meats (Juan-Pablo et al., 2000). Yeasts are potent consumers of lactic acid in foods which lead to an increase in the pH (Juan-Pablo et al., 2000; Walker, 1977). This was evident in CP2 and KS samples which had higher ($p \leq 0.05$) yeast counts and pH values than the other samples."

Sausages with a short ripening time have more lactobacilli right from the early stages of fermentation. In contrast, sausages with longer maturation times contain higher numbers of *Micrococcaceae* (Comi et al., 2005). *Micrococcaceae* and LAB are the most important microorganisms used as starter cultures in the processing of dry-fermented sausages. Micrococcaceae participate in the preservation of meat products, avoiding rancidness and developing the typical red colour due to catalase and nitrate

reductase (Martin et al., 2007). Some *Micrococcaceae* strains are able to produce antimicrobial substances. In this study, the high numbers of *Micrococcaceae* were found with values in the range of 2.70 x 10^4 to 7.50 x 10^5 cfu/g. This average value was slightly higher than those reported in sausages produced in the North East of Italy (Comi et al., 2005).

The enterococci also play an important role in the ripening of the sausages (Hugas et al., 2003), probably through proteolysis, lipolysis, and citrate breakdown, hence contributing to their typical taste and flavour and enhance colour stability, prevent rancidity and release various aromatic substances (Papamanoli et al., 2003). Enterococci have the ability to produce bacteriocins, the so-called enterocins, which are small peptides with antimicrobial activity towards closely related gram-positive bacteria including spoilage or pathogenic bacteria, such as *Listeria*. Moreover, enterococci are used in some countries as probiotics (Franz et al., 2003). In the present study, high counts of enterococci (>10^4 cfu/g) were found in Mhom, especially, samples from KK and CP2. The average enterococci counts were 1.60 x 10^5 cfu/g. This average value is slightly lower than those reported in Greek dry-fermented sausage (Papamanoli et al., 2003), sausages produced in the North East of Italy (Comi et al., 2005) and androlla, a Spanish traditional pork sausage (Fontan et al., 2007). Enterococci may be used as starter cultures in some foods and are commercially available as probiotic cultures for preventing and treating intestinal disorders in animals and humans (Stiles & Holzapfel, 1997). However, this genus may also be negatively associated with foods due to its possible implication as an indicator of faecal contamination (Franz, Holzapfel, & Stiles, 1999).

Staphylococcus aureus is an important foodborne pathogen in fermented meat products (Kang, & Fung, 2000). Production methods play a very important role for *S. aureus* to grow in dry and semi-dry sausage. For example, fermentation temperature applied at 30–40 °C gives possible growth of *S. aureus* and its enterotoxin production (Kaban, & Kaya, 2006). The first hours and days of fermentation are particularly critical. Since fermented sausage is not stable from the point of view of pH and A_w, complex microbiological activity occurs in the initial stage of fermentation (Kaban, & Kaya, 2006). Different types of fermented sausages have been implicated in staphylococcal food poisoning outbreaks. In this study, the

S. aureus counts were varied with values in the range of 3.3×10^3 to 2.7×10^6 cfu/g. The average value (5.7×10^5 cfu/g), and the individual values of each sample were within the wide range of *S. aureus* counts reported in the literature for different raw-cured sausages (Comi et al., 2005; Fontan et al., 2007). *S. aureus* could have contaminated the raw materials during the processing. This indicates the low standard of hygiene in the handling of the process and in the manufacturing plants. It is recommended that this product should be consumed as a cooked food.

However, the counts of coliforms were lower than 10^3 cfu/g whereas *E. coli* O157:H7, an important foodborne pathogen, was not detected in the tested samples.

Moreover, 71 isolates were obtained from MRS agar and 63 isolates were identified with the API-50 CH as lactobacilli (44 were identified as *Lactobacillus plantarum*, 3 as *Lactobacillus curvatus*, 4 as *Lactobacillus delbrueckii*, 4 as *Lactobacillus paracasei*, 2 as *Lactobacillus acidophilus*, 2 as *Lactobacillus brevis 1*, 2 as *Lactobacillus brevis 3*, 1 as *Lactobacillus pentosus* and 1 as *Lactobacillus mesenteroides*) with 88.73% of the extensively dominant isolates grown on MRS agar.

Lactobacillus plantarum was the main (61.97%) species of *Lactobacillus* in Mhom, especially in the sample from CP1 (50%). *L. plantarum*, together with *L. curvatus*, are the two lactobacilli species most commonly isolated from meat and meat products, including sausages elaborated with different technologies (Schillinger, & Lucke, 1987). *L. plantarum* has also been isolated in other raw-cured sausages (Coppola et al., 2000), but in larger proportion observed in this study. *L. delbrueckii* count presented a similar proportion to that of *L. paracasei*, accounted for 5.63% of the isolates. The total number of LAB species was the most abundant in CP1, followed by MK, CP2, KK and KS, respectively.

4. Conclusion

Mhom samples were collected from four different locations in the Northeastern regions of Thailand i.e., Chaiyaphum (CP), Khon Kaen (KK), Kalasin (KS) and Maha Sarakham (MK) provinces in order to determine their microbiological and hygienic qualities. These meat products are manufactured with traditional technologies without pure starter cultures. Their manufacture depends on the skill

and experience of the meat 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 flavour, texture, nutritional qualities, safety, and other characteristics of this type of sausage. The results showed high counts of total aerobic plate count, yeast and mould, LAB, *Micrococaceae* and *Enterobacteriaceae*. The LAB (above all homofermentative lactobacilli) was the main microbial group. *E. coli* O157:H7 was not detected in the products. The high count of *S. aureus* was observed; this indicates the low hygienic practices. It is recommended that this product should be consumed as a cooked food.

Further studies are necessary such as determination of the antimicrobial activity of the strains and their technological properties in Mhom production, considering the possible interactions among starter strains in the meat mixture.

Acknowledgments

This work has been funded by Commission on Higher Education and Postharvest Technology Innovation Center: KKU, and Agricultural Machinery and Postharvest Technology Research Center, KKU. We would like to gratefully acknowledge the staff of the Food Microbiology Laboratory, Department of Food Technology, Faculty of Technology, Khon Kaen University, Thailand, for their collaboration and providing laboratory facilities.

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CURRICULUM VITAE

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