CHAPTER III

MATERIALS AND METHODS

3.1 Study design

This study is an observational cross-sectional study which was conducted during November 2010 to April 2011. The cooked Northern Thai sausages were collected from Chiang Mai Province, Thailand. Microbiological examination was performed and compared with the microbiological criteria for Northern Thai sausage. The microbiological criteria consist of total colony counts for *Escherichia coli, Staphylococcus aureus, Clostridium perfringens*, yeast, and mold as well as the presence of *Salmonella* spp. in the Northern Thai sausage samples. Microsoft Excel 2007 program was used to determine the statistical difference between the microbiological result in the Northern Thai sausage sample and the acceptable limit criteria.

3.2 Sample size

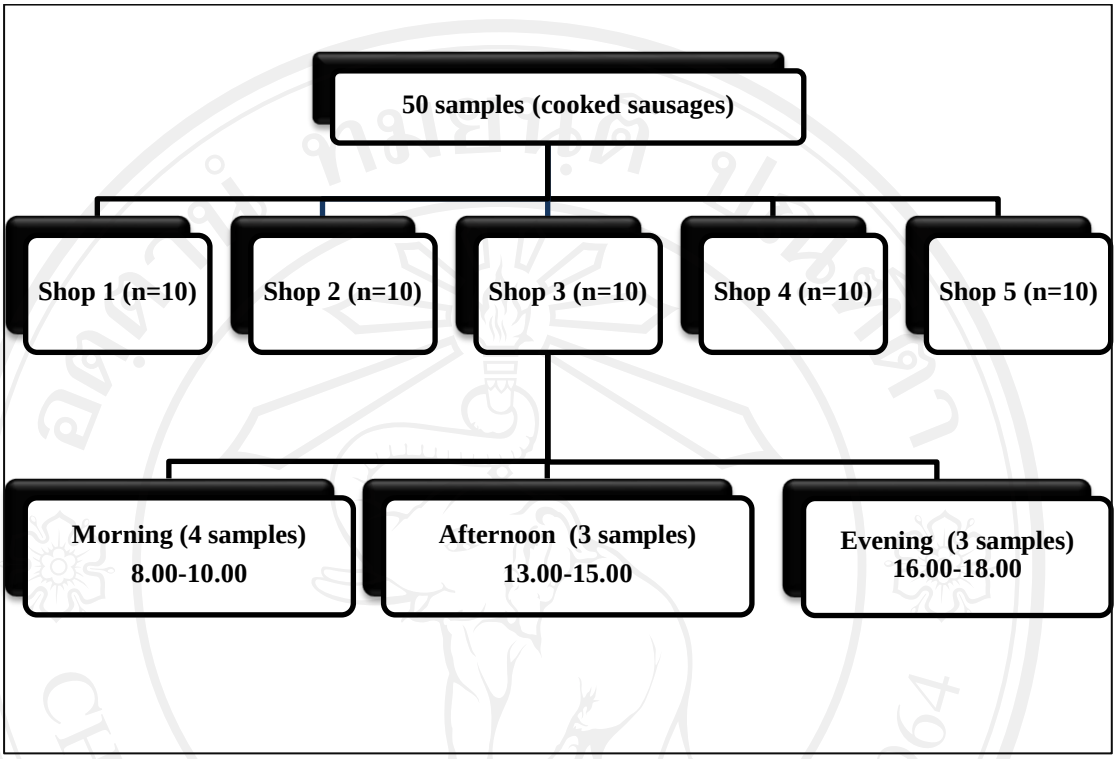
3.2.1 Population frame

The population frame of this study was 19 registered Northern Thai sausage shop from Chiang Mai provincial public health office database. The data avaliable from url:http://www.chiangmaihealth.com/cmpho_web53/index.php?option=com_content &view=frontpage&Itemid=7.

3.2.2 Sample size determination

Five Northern Thai sausage shops were selected. Ten cooked sausage samples were collected from each shop. The collecting was conducted in three rounds on different days: morning (8-10.00), afternoon (13.00-15.00), and evening (16.00-18.00). The sample sizes are shown in Figure 2.

Figure 2: Sample size distribution



3.3 Samples selection

3.3.1 Shop selection

The study population frame were 5 Northern Thai sausage shops.

3.4 Samples and data collection

3.4.1 Cooked Northern Thai sausage sample collection

Two hundred grams of cooked Northern Thai sausage was purchased from each of the selected shops. Information including shop name, date of collection, and time of collection were recorded. Samples were transported to the laboratory within two hours of collection. The sausage samples were kept at 4°C-8°C prior to further processing, and microbiological examination was conducted within 3 hours. The growth factors in this study include internal temperature, pH, and water activity. Temperature and pH measurments were made within 2 centimeters of the surface of

the sausages. Temperature and pH measurements were performed with a pH meter (pH300/310®, Oakton Instruments, Vernon Hills, USA). Water activity measurement was performed using a water activity measuring device (AquaLab series3® Decagon, Hopkins Court Pullman, USA). Laboratory procedures are shown in Figure 3. Microbiological examination followed International Organization for Standardization (ISO) methods as shown in Table 8.

Figure 3: Sample collection and laboratory examination procedures

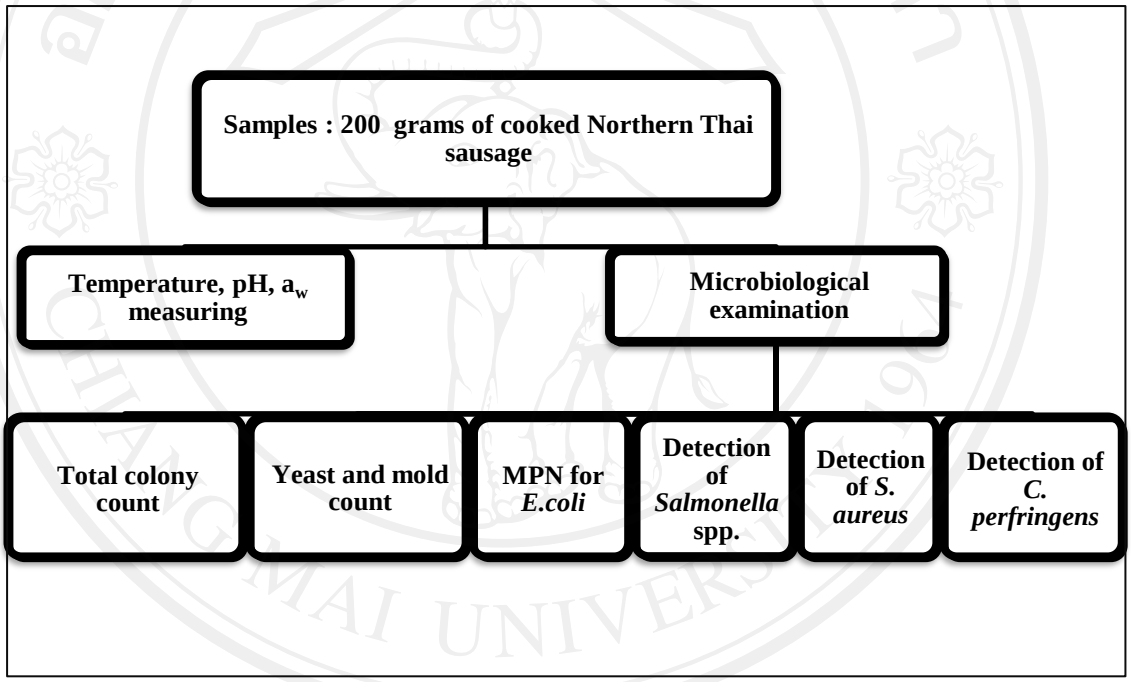


Table 8: Laboratory examination protocols

Methods	References
Total colony count	ISO 4833:2003
Most probable number for *Escherlichia coli*	ISO 4831:2006 and ISO 7251:2005
Detection of *Salmonella* spp.	ISO 6579:2002
Detection of coagulase-positive staphylococci	ISO 6888-1:1999
Detection of *Clostridium perfringens*	ISO 7397:2004
Total yeast and mold count	ISO 21527-1:2008

3.4.2 Data collection

The geographical data, manufacturer's names, dates of collection, growth factors (temperature, pH and water activity), and microbiology laboratory results were collected for statistical analysis.

3.4.2.1 Data on producers

Data on producers consisted of shop names, dates and times of collection, collection batch names, and sample numbers.

3.4.2.2 Growth factors data

Growth factors data consisted of internal temperatures, pH values, and water activity results.

3.4.2.4 Microbiological data

Microbiological data consisted of total colony counts (log cfu/g), total yeast and mold counts (log cfu/gram), most probable number for *Escherlichia coli* results (MPN/g), detection of *Salmonella* spp. results, detection of *Staphylococcus aureus* results, and detection of *Clostridium perfringens* results.

3.5 Laboratory procedures

3.5.1 Enumeration of microorganisms using the colony count technique

The method used in this study was modified from ISO 4833:2003. Ten gram samples were inoculated in 90 ml of maximum recovery diluents and homogenized with a stomacher for 1 minute. Decimal dilutions were prepared from 10^{-1} to 10^{-5} dilution. Then, each 0.1 ml of the dilution was spreaded to two sterile plate count agars (duplicate) for each dilution. The medium was left to dry. The dishes were incubated at 30°C ± 1°C for 72 hours ± 3 hours. The two successive dilution dishes that contained 15-300 colonies were counted and results calculated according to formula:

$$\mathbf{N} = \frac{\sum \mathbf{c}}{\mathbf{v}(\mathbf{n1} + \mathbf{0.1n2})\mathbf{d}}$$

N is number of Colonies present per milliliter or per gram of product

$\sum$**c** is the sum of colonies on all the dishes selected

V is the volume of inoculums on each dish in milliliters

$\mathbf{n_1}$ is the number of dishes selected at the first dilution

$\mathbf{n_2}$ is the number of dishes selected at the second dilution

d is the concentration of the first successive dilution plate

3.5.2 Enumeration of coliforms and *Escherichia coli* using most probable number technique

The method used in this study was modified from ISO 4831 and ISO 7251 methods. Ten gram samples were inoculated in 90 ml of maximum recovery diluents and homogenized with a stomacher for 1 minute. Decimal dilution was prepared from 10^{-1} to 10^{-5} dilution by steps.

One ml of initial suspension and following dilution was inoculated in 10 ml single-strength Lauryl sulfate tryptose broth. There were three tubes per dilution and the tubes contained Durham tubes. Ten ml of initial suspension and following dilutions were inoculated in 10 ml double-strength Lauryl-sulfate-tryptose broth. There were three tubes per dilution and the tubes contain Durham tubes. The inoculated tubes were kept at either 30°C ± 1°C or 37°C ± 1°C for 24 hours ± 2 hours.

After incubation, for single-strength Lauryl-sulfate-tryptose broth tubes, gas production was observed, and if neither gas formation nor opacity precluding the detection of gas formation was observed, incubation continued for another 24 hours ± 2 hours. Gas positive tubes were subjected to further comfirmation in lactose-bile-brilliant-green-broth (three tubes per dilution, containing Durham tubes).

One loop from the suspensions in any gas formation tube and in all cases from each tube of double strength Lauryl sulfate tryptose broth was inoculated in lactose-bile-brilliant-green-broth (three tubes per dilution, containing Durham tubes) for confirmation.

The inoculated lactose-bile-brilliant-green-broth tubes were kept at 30^0C±1^0C or 37^0C±1^0C for 24 hours±2 hours. A tube in which gas formation was observed after 24 hours±2 hours was considered to be a coliform positive tube.

The number of gas producing tubes were counted and compared with a most probable number (MPN) table. The positive coliform suspensions were further inoculated in EC broth (test tube containing a Durham tube) and incubated at 35^0C± 2^0C for 18-24 hrs. If gas production was observed, the tube was considered to be *Escherlichia coli* positive.

The biochemical test for *Escherlichia coli* was the indole test. One loopfull of each EC positive tube was inoculated into a tube containing tryptone or tryptophan medium and incubated at 37^0C±1^0C for 24 hours±2 hours. After incubation, 1 ml of Kovac's reagent was added to the tube. If a red ring was present, the results were indole positive and confirmed that *Escherlichia coli* was present in the sample.

3.5.3 Detection of *Salmonella* spp.

The method used in this study was modified from the ISO 6579 method. Twenty-five grams of sample were inoculated in 225 ml. of buffer peptone water and homogenized by stomacher for 1 minute, then the suspension was incubated at 37^0C± 1^0C for 18 hours ±2 hours. After incubation**,** 0.1 ml of suspension was inoculated in Rappaport-Vassilliadis medium with soya (RVS broth) and 1 ml of suspension was inoculated in Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn Broth).

Inoculated RVS broths were incubated at 41.5^0C±1^0C for 24 hours±3 hours and inoculated MKTTn broths were incubated at 37^0C±1^0C for 24 hours±3 hours.

After incubation, The inoculated RVS broth and inoculated MKTTn broth were further inoculated in Xylose Lisina Tergitol-4 agar (XLT-4 agar**)** and Brilliant Green Phenol Red Lactose Sucrose Agar (BPLS agar). The agars were incubated at 37^0C ± 1^0C for 24 hours ± 3 hours.

Typical colonies for *Salmonella* spp. in XLT-4 agar have a black center and a slightly transparent zone of reddish color due to the color change of the indicator. The typical colonies from BPLS agar were red.

Five typical colonies from the Xylose Lisina Tergitol-4 (XLT-4) agar and Brilliant Green Phenol Red Lactose Sucrose Agar (BPLS) agar samples were then selected and streaked on nutrient agar. Inoculated nutrient agar was incubated at 37^0C±1^0C for 24 hours ± 3 hours.

Pure cultures were selected for biochemical testing. Biochemical confirmation procedures consisted of carbohydrate fermentation and the production of hydrogen sulfite (TSI agar test), Urease test, Voges-Proskauer (VP) reaction test, and indole reaction test. The interpretation of biochemical tests are shown in Table 9 and table 10.

The pure colonies from Nutrient agar were tested by slide agglutination to determine the presence of Salmonella O-antigen, Vi-antigen, and H-antigen with the appropriate sera. The agglutination results were compared to The Kauffmann-White schem(table11).

Table 9: Biochemical reaction patterns in primary tests for the common clinically significant *Enterobacteriaceae (Jawetz et al., 1987).**

	Citrobacter	*Enterobacter*	*Escherlichia*	*Klebsiella*	*Morganella*	*Proteus*	*Providencia*	*Salmonella*	*Serratia*	*Shigella*
Arginine	±	±	-	-	-	-	-	±	-	-
Citrate	+	+	-	+	-	±	+	±	+	-
DNase	-	-	-	-	-	-	-	-	+	-
Gas	+	+	+	±	±	±	±	±	±	±
Glucose	+	+	+	+	+	+	+	+	+	+
H_2S	±	-	-	-	-	+	-	±	-	-
Indole	±	-	+	±	+	±	+	-	-	±
Lysine	-	±	+	+	-	-	-	+	+	-
Motility	+	+	±	-	+	+	+	+	+	-
Ornithine	±	+	±	-	+	±	-	+	+	±
Phenylalanine	-	-	-	-	+	+	+	-	+	-
Sucrose	±	+	±	+	-	±	±	-	+	-
Urease	-	-	-	±	+	+	±	-	-	-
VP*	-	+	-	+	-	-	-	-	+	-
TSI** slant	**A/K (A)**	**A**	**A (A/K)**	**A**	**A**	**A/K**	**A/K**	**A/K**	**A/K (A)**	**A/K**
TSI** butt	**AG**	**AG**	**AG**	**AG**	**AG**	**AG**	**AG**	**A;G±**	**A**	**A**

*Result for common clinical isolates: ± = **variable;** + = most(usually ≥ 90%) of straint positive; A= acid (yellow); G= gas; A/K= alkaline.(note: there are exceptional to nearly all of the results listed). VP* = Voges proskauer reaction. TSI**= Triple sugar iron aga

Table 10: Interpretation of biochemical test for *salmonella* spp. (Eley, 1996)

Test[a]	*Salmonella* strain									
	S.Thyphi		S.Parathyphi A		S.Parathyphi B		S.Parathyphi C		Other strains	
	Reaction	%[b]	Reaction	%[c]	Reaction	%[c]	Reaction	%[c]	Reaction	%[b]
TSI acid from glucose	+	100	+	100	+		+		+	100
TSI acid from glucose	-[d]	0	+	100	+		+		+	92
TSI acid from glucose	-	2	-	100	-		-		-	1
TSI acid from glucose	-	0	-	0	-		-		-	1
TSI hydrogen sulfite produce	+	97	-	10	+		+		+	92
Urea hydrolysis	-	0	-	0	-		-		-	1
Lysine decarboxylation	+	98	-	0	+		+		+	96
β-galactosidase reaction	-	0	-	0	-		-		-	2[e]
Voges-Proskauer reaction	-	0	-	0	-		-		-	0
Production of indole	-	0	-	0	-		-		-	1

a) From reference[5]

b) These percentages indicated that not all isolates of *Salmonella* serotypes show the reaction mark + or -. These percentages may vary between and within serotype of food poisoning serotype from different locations.

c) The percentages are not known from available literature.

d) *Salmonella Thyphi* is anaerogenic

e) The *Salmonella enterica* subspecies *arizonae* gave a positive or negative reaction but was always β-galactosidase positive. For the study of this strain it may be useful to carry out complementary examinations.

Table 11: The Kauffmann-White scheme of serological classification(Eley, 1996)

Serotype	O (somatic antigen)	H (Flagella antigen)	
		Phase 1	Phase 2
S.Parathyphi A	1,2,12	a	-
S.Parathyphi B	1,4,5,12	b	1,2
S.Thyphimurium	1,4,5,12	i	1,2
S.Parathyphi C	6,7,(Vi)	c	1,5
S.Thyphi	9,12,Vi	d	-
S.Enteritidis	1,9,12	g,m	-

3.5.4 Detection of coagulase-positive staphylococci using Baird-Parker agar medium techniques

The method for this study was modified from the ISO 6888-1 method. Ten gram samples were inoculated in 90 ml of Maximum Recovery Diluents. The inoculated suspension was homogenized in a stomacher for 1 minute. Decimal dilution was prepared from 10^{-1} to 10^{-5} dilution in steps. After that, each 0.5 ml of initial suspension was spread over of Baird Parker agar (four plate per dilution).

The plates were allowed to dry for 15 minutes and were incubated at 37^0C $\pm 1^0$C for 24 hours. Typical colonies from Baird Parker agar were black or grey, shining and convex. The colonies were 1 mm to 1.5 mm in diameter after incubation for 24 hours. They were surrounded by clear zone. Atypical colonies were shrunken and black with or without narrow white edges and the clear zone was absent or barely visible. Atypical colonies might present as grey colonies with no clear zone. Five typical colonies and 5 atypical colonies were selected and were inoculated in 5 ml brain heart infusion broth. The inoculated broth was incubated at 37^0C$\pm 1^0$C for 24 hours.

Plates with less than 150 colonies (in two successive dilutions) were selected for the coagulase test in which 0.1 ml of incubated brain heart infusion broth was inoculated in 0.3 ml of rabbit plasma in a sterile tube and incubated in 37^0C$\pm 1^0$C for 24 hours.

Coagulase positive results were expressed by clots occupying more than three-quarters of the origin volume of the liquid.

3.5.5 Detection of *Clostridium perfringens*

The method used in this study was modified from the ISO 4831 method. Ten gram samples were inoculated in 90 ml Maximum recovery diluents and homogenized by stomacher for 1 minute. Decimal dilution was prepared from 10^{-1} to 10^{-5} dilution in steps. One ml of the test sample was transferred into two sterile Petri dishes (duplicated). Ten to fifteen ml. of Tryptose-sulfite Agar with D-cycloserine (SC agar) at 44°C to 47°C were poured into the dishes. The inoculums and medium were gently mixed. The inoculums and agar were left to solidify for 15 minutes. Then another layer of 10 ml of the same agar was added. The inoculated dishes were kept at 37°C±1°C for 20 hours±2 hours under anaerobic conditions.

Typical *Clostridium perfringens* colonies are black in color. Five typical colonies were selected for inoculation into fluid thioglycollate medium and incubated under anaerobic conditions at 37^0C for 18 to 24 hours. After incubation, fives drops of thioglycollate medium was inoculated into lactose sulfite medium (contained Durham tubes). The tubes were incubated aerobically at 46^0C for 18 to 24 hours in a water bath.

Gas production and the presence of iron sulfite precipitation (a black color) was observed. Durham tubes more than one-quarter full of gas and tubes having more black precipitate were suspected *Clostridium perfringens* positive tubes.

For tubes where blackened medium was present, but where the Durham tubes were less than one-quarter full of gas, five drops of the medium were transferred to another tube of lactose sulfite medium. The lactose sulfite medium tubes contained Durham tubes. The inoculated lactose sulfite medium tubes were further aerobically incubated at 46^0C for 18 to 24 hours in a water bath.

Bacteria which form characteristic colonies in the tryptose-sulfite agar with D-cycloserine (SC) agar and which give a positive confirmation with the lactose sulfite medium were considered as being *Clostridium perfringens* positive in the sample.

3.5.6 Enumeration of yeasts and molds using colony count techniques

Ten grams of sample were inoculated in 90 ml maximum recovery diluents and homogenize by Stomacher for 1 minute. Decimal dilutions were prepared from 10^{-1} to 10^{-5} dilution by steps. 1 ml of each dilution step was spreaded on yeast extract-dextrose-chloramphenical agar. The dishes were left to dry and incubate at $25^0C \pm 1^0C$ for 5 days. Colonies on each dish were counted after 3, 4, and 5 days of incubation. After 5 days, the dishes containing fewer than 150 colonies were retained. If well isolated colonies were difficult to count or overgrowths of molds on plates were found, the colony number results were recorded from day 4 or day 3 of incubation. The two lowest dilution step plates which contained 10 to 150 colonies were selected and calculations completed using the formula:

$$\mathbf{N} = \frac{\sum \mathbf{c}}{(\mathbf{n1} + \mathbf{0.1n2})\mathbf{d}}$$

$\sum \mathbf{c}$ is the sum of the colonies counted on all the plates

$\mathbf{n_1}$ is the number of plates counted in the first dilution

$\mathbf{n_2}$ is the number of plates counted in the second dilution

d is the dilution from which the first counts were obtained

3.6 Statistical data analysis

The results, consisting of total colony count number and total yeast and mold count number, were checked for normal distribution. The values were transformed to log numbers for statistical testing. In cases where *Escherlichia coli* were not found in the sausage samples, the MPN number used was 50% of the detection limit or 0.15 MPN/g. Descriptive statistics for each sample were calculated.

The percentage of sausage samples which achieved the standard were calculated by comparing with Thai community product standard criteria.

Data were further analyzed using Microsoft Excel version 2007 program. One-way ANOVA was used to compare differences between each growth factor and the colony forming unit number among the three groups. The Kruskal-Wallis rank test was used to compare differences among the three groups in cases where the variances were unequal.

The growth factors, including temperature, water activity, and pH, were compared with the optimal growth range of each microorganism. Microbiological results of each sample were interpreted using the microbiological standard level.

By Microsoft Excel, the condition “pass” was given to sausage samples where the microbiological result met the Thai community product standard criteria, and “Not pass” for sausage samples that microbiological result were not achieve Thai community product standard criteria. The condition “Support growth” were gave to sausage samples where all study growth factors (temperature, water activity, and pH) were in the same ranges of microorganisms. The condition “Not support growth” were gave to sausage samples which their growth factor were not met at least one of study growth factor. The number of sausage sample in each condition were counted. Percentage of sausage which met the standard criteria and percentage of sausage which have growth factor in the same range as growth range of microorganisms were calculated.