CHAPTER III
MATERIALS AND METHODS

3.1 Comparison of *E. coli* and *Salmonella* spp. detection methods

The experiment was performed in completely randomized design (CRD) with 3 replications to compare 3 detection methods; conventional method (FDA/BAM), 3M Petrifilm E.coli/Coliform Count plates (AOAC (Petrifilm)) and RT-PCR (modified from O'Hanlon *et al.* (2004) and Malorny *et al.* (2004)). For testing of pure culture, plate count method was added. All methods were used for determination of *E. coli* in agricultural samples except plate count technique. Determination of *Salmonella* spp. was compared between conventional method (FDA/BAM) and RT-PCR only.

3.1.1 Enumeration of pure culture

E. coli ATCC 25922 (Lot. 1896) and *S. typhimurium* ATCC 13311(Lot. 1894) from Department of Medical Science, Ministry of Public Health of Thailand (Nonthaburi, Thailand) was used as positive control. *E. coli* ATCC 25922 was enriched with 25 mL of Lauryl Sulfate broth (LST) (Merck, Darmstadt, Germany). LST broth composed of 20 g tryptose or trypticase, 5 g lactose, 2.75 g K_2HPO_4, 2.75 g KH_2PO_4, 5 g NaCl, 0.1 g sodium lauryl sulfate, and 1 L distilled water and adjusted pH to 6.8±2 with 1M HCl or 1M NaOH. Medium was steriled at 121°C, 15 pound per square inch pressure for 15 min. It was incubated at 37±1°C for 24 h. Initial quantity of *E. coli* ATCC 25922 was evaluated by the plate count technique. *E. coli* culture was serially diluted in 10-fold with distilled water. Aliquots (1 mL) from the 10^{-1} to 10^{-9} distilled water serial dilutions were spread on Levine's Eosin-Methylene Blue agar (L-EMB) (Merck, Darmstadt, Germany). L-EMB agar was composed of 10 g peptone, 10 g lactose, 2 g K_2HPO_4, 15 g agar, 0.4 g eosin Y, 0.065 g methylene blue and 1 L distilled water. Final pH was 7.1±0.2 adjusted with 1M HCl or 1M NaOH. All ingredients were boiled and dispensed 200 mL portions in bottle. It was autoclaved at 121°C for 15 min. The plates of L-EMB were incubated at 37±1°C for 24 h and number of colony forming units (CFU) was counted.

S. typhimurium ATCC 13311(Lot. 1894) was enriched in trypticase soy broth (TSB) (Lab-Scan, Bangkok, Thailand). TSB broth was composed of 17 g trypticase peptone, 3 g phytone peptone, 5 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose and 1 L distilled water. Final pH was 7.3±0.2 adjusted by 1M HCl or 1M NaOH. The broth was sterilized at 121°C with 15 pound per square inch for 15 min. The quantity of bacteria was 10-fold serially diluted and determined by plate count using Xylose lysine desoxycholate (XLD) (Merck, Damstadt, Germany). Plates were incubated at 37±1°C for 24±2 h. Ingredients of XLD agar were 3 g yeast extract, 5 g L-lysine, 3.75 g xylose, 7.5 g lactose, 7.5 g sucrose, sodium 2.5 g desoxycholate, 0.8 g ferric ammonium citrate, 6.8 g sodium thiosulfate, 5 g NaCl, 15 g agar, 0.08 g phenol red and 1 L distilled water. Final pH was 7.4±0.2, it was not store more than 1 day and non-autoclave.

Then, *E. coli* ATCC 25922 determination was compared by 3 methods: conventional, 3M Petrifilm and RT-PCR methods, each method was made in triplicate. Whereas, *S. typhimurium* ATCC 13311, quantity was compared between conventional and RT-PCR method only.

3.1.1.1 Conventional method

Conventional method for *E. coli*

This procedure followed the method in BAM (Bacteriological Analytical Manual) (Feng *et al.*, 2002). One-mL aliquot of each serial dilution from 10^{-1} to 10^{-3} was inoculated in the three fermentation tubes of LST broth. Each of which was inserted internally with Durham tube for gassing observation. LST tubes were incubated at 35±0.5 °C for 24±2 h. More 24±2 h with the same condition was performed, unless the gassing was not observed in LST tube. Consequently, a loop full (10 µL) of each suspension from the positive tube was transferred into fermentation tubes which contained 8 mL of the Escherichia coli broth (EC) (Merck, Darmstadt, Germany). EC broth was composed of 20 g trypticase or tryptose, 1.5g bile salts No.3, 5 g lactose, 5 g K_2HPO_4, 1.5 g KH_2PO_4, 5 g NaCl and 1 L distilled water. Final pH was 6.9±0.2 adjusted by 1M HCl or 1M NaOH and autoclaved at 121°C with 15 pound per square inch pressure for 15 min. EC tubes were incubated at 45.5±0.5°C for 24±2 h and then determination was made. The complete test for *E. coli* was done

by streaking one loop full of positive EC tubes on L-EMB agar. The plate of L-EMB agar was incubated at 35±1°C for 24±2 h. Finally, the *E. coli* colonies appeared in dark centered and flat, with or without metallic sheen were examined and recorded for a positive result.

Conventional method for *Salmonella* spp.

Again, this procedure followed the method in BAM (Bacteriological Analytical Manual) (Andrews and Hammack, 2007). Tetrathionate broth base (TT) (Merck, Damstadt, Germany) was prepared as follow: mixed suspended ingredients were 5 g polypeptone, 1 g bile salts, 10 g calcium carbonate, 30 g sodium thiosulfate.$5H_2O$ in 1 L distilled water and heated to boiling. It was not autoclaved. Then it was cooled down, then 20 mL of iodine-potassium iodide solution (I_2-KI) (5 g potassium iodide, 6 g iodine and 20 mL distilled water) and 10 mL brilliant green solution were added. Rappapor-Vassiliadis Medium (RV) (Merck, Damstadt, Germany) was combined with 1000 mL broth base (5 g tryptone, 8 g NaCl, 1.6 g KH_2PO_4 and 1L distilled water), 100 mL magnesium chloride solution (400 g $MgCl_2.6H_2O$ and 1 L distilled water) and 10 mL malachite green oxalate solution (0.4 g malachite green oxalate and 100 mL distilled water). Total volume of complete medium was 1110 mL. Ten mL volume of complete medium was dispensed into 16x150 mm test tubes. It was autoclaved for 15 min at 115°C. Final pH was 5.5 ±0.2. Bismust sulfite (BS) agar (Merck, Damstadt, Germany) mixed and heat ingredients were 10 g polypeptone (or peptone), 5 g beef extract, 5 g dextrose, 4 g Na_2HPO_4 (anhydrous), 0.3 g $FeSO_4$ (anhydrous), 8 g bismuth sulfite (indicator), 0.025 g brilliant 20 g green, agar and 1 L distilled water. These were boiled about 1 min, then it was cooled to 45-50°C, poured 15-20 mL in to sterile 15x100 mm petri dishes and let the medium plates dry about 2 h with lids partially remove; then closed the plates. Final pH was 7.7±2. It was not autoclaved and plates should be prepared one day before streaking and store in the dark. Selectivity would be loss after 48 h. Hektoen enteric (HE) agar (Merck, Damstadt, Germany) contains 12 g peptone, 3 g yeast extract, 9 g bile salts No.3, 12 g lactose, 12 g sucrose, 2 g salicin, 5 g NaCl, 5 g sodium thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g bromthymol blue, 0.1 g acid fuchsin, 14 g agar and 1 L distilled water (pH 7.5±0.2). These ingredients were boiled to dissolve all components and the amount of 15-20 mL

solution was poured into each sterilized 15x100 mm petri dish. This method recommended that the medium must be used within 1 day after completely preparing.

One mL of *S. typhimurium* ATCC 13311 cultured broth was mixed to 10 mL TT broth and 0.1 mL to 10 mL RV medium. Both media were incubated at 42±1°C for 24±2 h. Isolation of *Salmonella* spp. from both media was done by streaking a loopful (10μL) on BS, XLD and HE agar. These agar plates were incubated at 35±1°C for 24±2. The colony morphology characters of bacteria grown on selective media was determined by brown, gray or black with sometimes a metallic sheen performing on BS agar, pink with or without black centers or black colony on XLD agar, blue-green to blue colony with or without black centers or black on HE agar.

3.1.1.2 3M Petrifilm method for *E. coli*

E. coli counts were determined in 3M Petrifilm™ *E.coli* count plates. One-mL aliquot of each serial dilution 10^{-1} to 10^{-9} of *E. coli* culture were spread on 3M Petrifilm™ *E.coli* count plates (3M, America). The inoculated plates were incubated at 35±1°C for 48±2 h. On the 3M Petrifilm™ *E.coli* count plates, the blue colonies with gas bubbles were counted as typical *E. coli* (Matner *et al.*, 1990).

3.1.1.3 RT-PCR method

a. DNA extraction

Genomic DNA was extracted from both cultures, *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311. An aliquot of 1 mL of 10^{-1} from each pure culture was transferred to 1.5 mL microcentifuge tube. After centrifugation at 12,000 rpm at 4°C for 5 min, the supernatant was discarded and the pellet was washed twice with 1M NaCl. Five hundred μL of TE buffer was pipetted into the microcentrifuge tube, vortex to resuspend and centrifuge at 12,000 rpm at 4°C for 5 min. The supernatant was discarded and add 500 μL TE buffer with 0.1% Tween20 were added, and heat at 90°C for 10 min by heat block. The mixed suspension was centrifuged at 12,000 rpm at 4°C for 5 min. The supernatant was then, collected and transferred into a new tube. This supernatant was used as template DNA.

b. Standard curve

The standard curve was modified from An *et al.* (2006): *E. coli* ATCC 25922 (Lot.1896) was enriched in LST broth, incubated at 35±2 °C for 18±2 h. The freshly

prepared *E. coli* ATCC 25922 (Lot.1896) was harvested and serially diluted in 10-fold with distilled water (10^{-1} to 10^{-9}). Aliquot of 1 mL from the 10^{-5} to 10^{-9} of each serial dilution was spread plate in L-EMB agar and made triplicate. The bacterial quantity was enumerated after plates were incubated at 37±2 °C for 24±2 h. Its DNA extracted from serial dilution of 10^{-5} to 10^{-9} were followed the DNA extraction in 3.1.1.3 a. The standard curve of *S. typhimurium* ATCC 1311 (Lot. 1894) was done as same as those of *E. coli* but it was enriched in TSB broth.

c. Optimization of RT-PCR condition

Preparing the PCR conditions, the template DNA was (2μL) mixed with 10 μL of 1X DyNAmoTM HS SYBR Green qPCR kit (Finnzymes, ESpoo, Finland). One μL of 0.5 μM of each primer for *E. coli* with forward primer of 5'-CATGCCGCGTGTATGAAG AA-3' (395 to 414 base pairs) and reverse primer of 5'-CGGGTAACGTCAATGAGC AAA-3' (470 to 490 base pairs); (Huijsdens *et al.*, 2002) was added with H_2O to a total volume of 20 μL for reaction. A 20 μL reaction mixture was transferred to RT-PCR tube (Bio-Rad laboratory, Hercules, CA, USA). *E. coli* primers from Huijsdens *et al.*, (2002) were based on 16s rDNA sequences, available in the National Center for Biotechnology Information databases. Alignment of 16S rDNA sequences with those from closely related bacteria revealed sequences specific for species. The sequence of the primer combination optimal for species was selected from these specific sequence detectors (Applied Biosystems, Foster City, California). To check for specificity, the selected primer target sites were compared to all available 16s rDNA sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST). They were found that a very high sensitivity: as little as 1 CFU of *E. coli* could be detected. Furthermore, the specificities of primer most closely related to *E. coli* and no PCR inhibition. Primers for *Salmonella* were ttr-6 (forward) 5'-CTCACCAGGAGAT TACAACATGG-3' and ttr-4 (reverse) 5'-AGCTCAGACCAAAAGTGACCATC-3' from Malorny *et al.* (2004), which is target within the ttrRSBCA locus and it is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. It is required for tetrathionate respiration in *Salmonella*. The specificity of the sequences was tested by a BLAST search in GeneBank, located at the National Center for Biotechnology Information website (BLAST version 2.0).

The diagnostic accuracy was shown to be 100% compared to the traditional culture methods from tested in 110 various food samples were investigated for *Salmonella*.

The negative control preparation was as same as the samples but without primers and template DNA. The RT-PCR tubes were covered with flat cap strips, and they were centrifuged briefly to bring all reagents to the bottom. Amplification reaction was performed using the following program with CHROMO4 RT-PCR instrument (Bio-Rad laboratory, Hercules, CA, USA): 10 min at 95 °C; 40 cycles of (10 s at 94°C, 30 s at 60°C, 30 s at 72°C, followed by a 5 min extension at 72°C and a hold at 4°C). Data analysis was performed using CHROMO4 RT-PCR detection system software. After completing the PCR amplification cycles, a melt curve was generated for the resulting loss of fluorescence over a temperature range of 55-92 °C. In this curves, negative first derivative plot is presented as the rate of change in fluorescence over temperature range. This graph represents the desired amplicon as distinct melting peak with specific melting temperature (T_m). If there contamination during the reaction, a peak, other than desired amplicon peak, would appear in melting curve thus indicating the contamination of DNA, non-specific binding, or occurrence of primer dimmer.

3.1.2 Enumeration of *E. coli* contaminated in agricultural samples

3.1.2.1 Sample collection

Samples were collected from four provinces in Thailand; Kanchanaburi, Phisanulok, Nakhonsawan and Chiang Mai. The choice was based on the fact that these areas are major producers of vegetables for both domestic consumption and export. In these four provinces, samples were collected from wholesale and retail markets, shelves, plantations and farms and Center of Agricultural Research (100 samples). Vegetable samples were collected from wholesale market, retail market and fresh vegetables in the shelf in Chiang Mai Province. Vegetables were in the list of export vegetables indicated by the Department of Foreign Trade. They consisted of 23 species including of coriander, stink water, holy basil, sweet basil, ka-yang, peppermint, puk-peaw, green shallot, Chinese celery, leave and flower of Chinese chive, *Acacia pennata*, lemon glass, convolvulus, water fern, water mimosa, centella, piper samentosum, amaranth, yard long bean, asparagus, chili pepper, and ceylon spinach. These of which were announced by the Department of Foreign Trade

(Thailand) have to be detected for *E. coli* contamination before export. The samples of soil, water, organic fertilizers, and feces amounting to 32, 17, 9 and 16 samples, were additionally collected respectively (Table 5).

Table 5 Agricultural samples collected at various locations.

No.	Type of samples	Detail of location	Note
1	peppermint	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
2	convolvulus	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
3	ceylon spinach	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
4	*Acacia pennata*	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
5	Chinese chive leave	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
6	green shallot	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
7	sweet basil	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
8	holy basil	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
9	coriander	Muang Mai market, Tambon Chang, Moi Amphoe Muang, Chiang Mai Province	
10	yard long bean	Muang Mai market, Tambon Chang Moi ,Amphoe Muang, Chiang Mai Province	
11	ka-yang	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
12	Chinese celery	Muang Mai market Tambon Chang Moi Amphoe Muang Chiang Mai Province	
13	lemon glass	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
14	leech lime leave	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
15	asparagus	Suthep market ,Tambon Suthep, Amphoe Muang, Chiang Mai Province	
16	centella	Suthep market, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
17	lettuce	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
18	stink weed	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
19	*Piper sarmentosum*	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
20	water mimosa	Muang Mai market, Tambon Chang Moi, Amphoe Muang, Chiang Mai Province	
21	common bean, green bean	Doi Kham Royal Project Foundation, Chiang Mai University, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
22	baby corn	Doi Kham Royal Project Foundation, Chiang Mai University, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
23	Chinese chive leave	Doi Kham Royal Project Foundation ,Chiang Mai University, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
24	*Piper sarmentosum*	Doi Kham Royal Project Foundation, Chiang Mai University, Tambon Suthep, Amphoe Muang, Chiang Mai Province	
25	green shallot	Doi Kham Royal Project Foundation, Chiang Mai University, Tambon Suthep, Amphoe Muang, Chiang Mai Province	

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
26	water	M.10 Bankhondoo, Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	tap water
27	water	Mr. Khien Somphee, 73 M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	well water
28	water	Mr. Khien Somphee, 73 M 2 Tambon Wangmahakron Amphoe Tatako Nakhonsawan Province	tap water
29	water	Miss Tuangjai Thongchu, Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	irrigation canal
30	soil	Mr. Khien Somphee, 73 M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	soil from stink weed field
31	soil	Mr. Kuan Thongchurah, 96 M 10 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	soil from chinese cabbage field
32	pig faces	Mr. Kuan Thongchurah, 96 M 10 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	dry pig faces
33	pig faces	Mrs.Sumahng Goonlawong, M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	dry pig faces
34	cow faces	Mrs. Seerut Suwannarit, M 9 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	fresh cow faces
35	chicken faces	Mrs. Duangsawan Daukdin, M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	
36	chicken faces mixed paddy husk	Mrs. Duangsawan Daukdin, M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	
37	cow faces	Mrs.Sumahng Goonlawong, M 2 Tambon Wangmahakron, Amphoe Tatako, Nakhonsawan Province	
38	water	Irrigated Agricultural Research Station, Faculty of Agriculture, Chiang Mai University	
39	water	Irrigated Agricultural Research Station, Faculty of Agriculture, Chiang Mai University	

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
40	soil	Irrigated Agricultural Research Station, Faculty of Agriculture, Chiang Mai University	soil from kale field
41	soil	Irrigated Agricultural Research Station Faculty of Agriculture Chiang Mai University	soil from chinese cabbage field
42	soil	Irrigated Agricultural Research Station, Faculty of Agriculture, Chiang Mai University	soil from pak choi field
43	organic fertilizer	Irrigated Agricultural Research Station, Faculty of Agriculture, Chiang Mai University	
44	water	Division of Vegetable Technology, Maejo University	water from channel of water
45	water	Division of Vegetable Technology, Maejo University	tap water
46	soil	Division of Vegetable Technology, Maejo University	soil from angled gourd field
47	soil	Division of Vegetable Technology, Maejo University	soil from sweet basil field
48	soil	Division of Vegetable Technology, Maejo University	soil from lettuce cos
49	soil	Mrs. Komkai Juntima, 80/35 Tambon Ngaungharn, Amphoe Sunsai, Chiang Mai Province	
50	organic fertilizer	Division of Vegetable Technology, Maejo University	
51	soil	Division of Vegetable Technology, Maejo University	soil from chili field
52	water	Mae Hia Agricultural Research Station and Training Center	tap water
53	water	Mae Hia Agricultural Research Station and Training Center	

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
54	soil	Mae Hia Agricultural Research Station and Training Center	soil from water spinach field
55	soil	Mae Hia Agricultural Research Station and Training Center	soil from carrot field
56	soil	Mae Hia Agricultural Research Station and Training Center	soil from broccoli field
57	soil	Mae Hia Agricultural Research Station and Training Center	soil from vegetable field
58	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
59	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
60	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
61	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
62	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
63	organic fertilizer	Mae Hia Agricultural Research Station and Training Center	
64	chicken faces	Mr. Wichien Saouy, 45 M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
65	soil	Mrs.Tanaum Junsetong, M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	soil from chinese cabbag field
66	soil	Mrs.Tanaum Junsetong, M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	soil from mustard green field
67	soil	Mrs.Tanaum Junsetong, M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	soil from pak choi field

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
68	cow faces	Mrs. Saibooah Toomsook, Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
69	cow faces	Mrs. Saibooah Toomsook, Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
70	cow faces	Mrs.Tanaum Junsetong, M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
71	cow faces	Mr. Wichien Saouy, 45 M1 Tambon Nakhonpamahk Amphoe Bangkratum Phitsanulok Province	
72	pig faces	Mr. Wichien Saouy 45 M1 Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
73	chicken faces	Mrs. Saibooah Toomsook ,Tambon Nakhonpamahk, Amphoe Bangkratum, Phitsanulok Province	
74	soil	Kanchanaburi Horticultural Research Center	soil from chili field
75	soil	Kanchanaburi Horticultural Research Center	
76	organic fertilizer	Kanchanaburi Horticultural Research Center	
77	soil	Kanchanaburi Horticultural Research Center	soil from okra field
78	soil	Kanchanaburi Horticultural Research Center	soil from asparagus field
79	soil	Kanchanaburi Horticultural Research Center	soil from sweet basil field
80	water	Kanchanaburi Horticultural Research Center	water from Kwai Noi river
81	water	Kanchanaburi Horticultural Research Center	underground water
82	soil	Mrs.Kosoom Bausa-aht, Tambon Tamakarm, Amphoe Muang, Kanchanaburi Province	soil from chili field

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
83	water	Mrs.Kosoom Bausa-aht, Tambon Tamakarm, Amphoe Muang, Kanchanaburi Province	
84	soil	Mr.Chumpon Puntumi,t Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	soil from yard long bean field
85	water	Mr.Chumpon Puntumit, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	
86	soil	Mr.Chumpon Puntumit, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	soil from angled loofah field
87	soil	Mrs.Sopaporn Sakarin, Tambon Tamakarm, Amphoe Muang, Kanchanaburi Privince	soil from brenjal field
88	water	Mrs.Sopaporn Sakarin, Tambon Tamakarm, Amphoe Muang, Kanchanaburi Privince	
89	soil	Mrs.Aroon Dubkaow, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	soil from yard long bean field
90	water	Mrs.Aroon Dubkaow, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	
91	soil	Mr.Umnart Mekpun, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	soil from goa bean field
92	soil	Mr.Umnart Mekpun, Tambon KowPoon, Amphoe Muang, Kanchanaburi Province	soil from okra field
93	water	Mr.Umnart Mekpun Tambon, KowPoon, Amphoe Muang, Kanchanaburi Province	
94	soil	Mr.Sonpong Mekdang, Tambon Wangyen, Amphoe Muang, Kanchanaburi Province	soil from baby corn field
95	soil	Mr.Sonpong Mekdang, Tambon Wangyen, Amphoe Muang, Kanchanaburi Province	soil from asparagus field
96	goat faces	Amphoe Muang, Kanchanaburi Province	

Table 5 Agricultural samples collected from various locations (continued).

No.	Type of samples	Detail of location	Note
97	cow faces	Mr.Chalau Maliwan, 14 M 4 Tambon Ngaungyah, Amphoe Muang, Kanchanaburi Province	
98	soil	Mrs.Sonjean Juntasoot 124/3 M 1 Tambon Ngaungyah Amphoe Muang Kanchanaburi Province	soil from yard long bean field
99	cow faces	Mr.Wichien Naak-on, 273/4 M 4 Tambon Ngaungyah, Amphoe Muang, Kanchanaburi Province	
100	celery	Doi Kham Royal Project Foundation, Chiang Mai University, Tambon Suthep, Amphoe Muang Chiang Mai Province	

3.1.2.2 Serial dilution preparation

Vegetable samples were cut to 1 cm to made them smaller by using sterile scissor or a knife then mixed. Soils, organic fertilizer and animal feces were not cut because these were small already. These samples were divided to 50 g, put into the stomached bag (Stomached 400 mL, Bibby Sterilin, Staffs, United Kingdom) containning 450 mL of Butterfield's phosphate buffer. For water samples, 1 mL was used to mixed in 9 mL of Butterfield's phosphate buffer (BPB) as 10^{-1}. The mixture was shaken for 10 min by shaker (shaken back and forth) (Yamato Shaker SA31, Tokyo, Japan) before 10-fold serial dilution, procedure was proceed with BPB from 10^{-2} to 10^{-9} . For detection of *Salmonella* 25 g sample was used to mix in 225 mL TSB and remained about 5 to 10 min before detection. Monitoring of *Salmonella* did not need serial dilution, only need 10^{-1} diluted sample.

3.1.2.3 Enumeration of *E. coli* and *Salmonella* spp. from agricultural samples

All samples were tested with 3 methods for *E. coli* while *Salmonella* spp. was tested with only 2 methods. Steps of enumeration were the same as pure culture but solution was sample solution.

3.1.3 Sensitivity and specificity

To determine the sensitivity and specificity of a particular test for detection of *E. coli* and *Salmonella* spp. the BAM method was used as a reference. Sensitivity (true positive rate) was calculated by dividing the number of samples positive by the test evaluated and positive by the reference method by the number of samples positive by the reference method. Specificity (true negative rate) was calculated by dividing the number of samples negative by the test evaluated and negative by the reference method by the number of samples negative by the reference method (Horman and Hanninen, 2006 and Genc *et al.*, 2005).

3.1.4 Statistical analysis

All data counts were converted to $\log_{10}$ count before statistical analysis. All data were analyzed by satitistix 8.0 (Tallahassee, FL, USA). Data were analyzed by analysis of variance (ANOVA) using a completely randomized design. The differences between the treatments were examined by Fishers least significant difference (LSD) test at a significance level of 95%. The correlation coefficient for methods was analyzed Pearson correlation coefficient.

3.2 Effect of composting animal manure on dynamic population of *E. coli* and *Salmonella* spp.

3.2.1 Experimental setup

The experiment was conducted in RCB design (Randomized Complete Blocks Design) with 4 treatments and 3 replications during June to October 2009 at Mae Hia Agricultural Research Station and Training Center. Each treatment was as follow:

1. Poultry layer manure composting
2. Poultry layer packed in the plastic bags (fertilizer sacks)
3. Cow manure composting
4. Cow feces packed in the plastic bags (fertilizer sacks).

3.2.2 Materials handling

Composting for poultry layer and cow manure was performed, using windrow composting system. Two materials were arranged in 2.2 m long 1.7 wide and 0.7 m high. This size of composting pile was easy for the maintenance of aerobic condition. Too small windrows will not attain the required temperatures for efficient composting and the destruction of pathogen (Susangkal and Chaerul, 2009). The optimum

moisture levels of both materials were about 60 to 70% by weight by spraying of water and mixed thoroughly (Muenchang *et al.*, 2005). The materials were turned over every 15 days and humidity in the piles were always maintained the same as beginning. Turning the compost pile ensure for enough air circulation through to all parts of the pile to encourage a quick decomposition rate.

Non-composting for poultry layer and cow feces was performed by packing in the plastic bag (fertilizer sacks), size 50x90 cm, with excluded area about 20 cm for tying the bag. Weight of poultry layer was about 30 kg/bag and 40 kg/bag for cow feces. These treatments were not adjusted for moisture. Three bags were made for one replication and they were placed under shading. This method imitated agriculturist's methods.

3.2.3 Isolation and enumeration of *E. coli* and *Salmonella* spp.

Poultry layer and cow manure compost was sampled by collecting from the middle and at the rim sides around the piles with approximately 30 to 40 cm deep. Whereas, the non-composted of those were collected from the packed bags the same method as the composted pile but the depth was about 20 to 30 cm. Sample collections of treatments were periodically determined at 1, 3, 5, 7, 14, 21, 28, 42, 56, 70, 84, 98, 112, 126 and 140 days. Each of the collected samples were thoroughly mixed before being tested. Enumeration of *E. coli* and isolation of *Salmonella* spp. followed BAM (Bacteriological Analytical Manual) (Feng *et al.*, 2002; Andrews and Hammack, 2007) as described in 3.1.1.1.

3.2.4 Change in quality of materials

These samples were remainder sample from 3.2.3. The samples were analyzed followed the methods of Department of Agriculture (2005), which was the method for analysis of organic fertilizer. C:N ratio, EC, OM, pH and temperature were determined as the parameters quality of both composted and non-composted manures.

3.2.5 Statistical analysis

The population of bacteria found in manure samples was converted to $\log_{10}$ count before statistical analysis. All recorded data were analyzed by satitistix 8.0 (Tallahassee, FL, USA). Analysis of variance (ANOVA) using a completely randomized block design was used for determination the differences between treatments by Fishers least significant difference (LSD) test at a significance level of

95%. The correlation coefficient for methods was calculated by Pearson correlation coefficient.

3.3 Reduction of contaminated of *E. coli* and *Salmonella* spp. by application of composted cow manure in export vegetables

3.3.1 Experimental setup

The experimental was conducted in split plot block design with triplicates. The main plots were the type of vegetables while those of subplots were type of manures. The main plots included asparagus, kale, coriander, stink weed and peppermint. The subplots were application of composted cow manure, application of non-composted cow manure and without application of manure. Both cow manures treatments were applied at a rate of 2 kg/m^2. The composted cow manure showed no contamination of *E. coli*, whereas the treatment of non-composted cow manure contaminated with *E. coli* by 6.32 log MPN/g. The experiment was carried out during January to April 2010 at Mae Hia Agricultural Research Station and Training Center. Before starting the experiment, the soil and water in the field were collected to detect the contamination of *E. coli* and *Salmonella* spp. followed the method in BAM (Feng *et al.*, 2002; Andrews and Hammack, 2007) as described in 3.1.1.1.

3.3.2 Plants bed preparation

Plant beds were carried out in 1x5 m for growing kale, coriander, stink weed and peppermint. Beds size for asparagus was 0.5x5 m. The composted and non-composted cow manure were dressed on the soil with 2 kg/m^2 and mixed well with harrow. The soil was watered and kept moisture before planting.

3.3.3 Vegetable production

Asparagus

The seed of cultivar Brock' improve was used seedling tray Asparagus. The seeds were soaked in the water over night, then transferred to seedling tray by using culture media containing soil: rice husk: coconut husk: burn-rice husk ratio 1: 1: 1: 1. The trays were regularly watered for one month, after that the seedlings were transferred to the growing bag, size of 10x15 cm with the same cultivating materials. Seedlings were fertilized with 46-0-0; N-P_2O_5-K_2O solution of 20g/L at the rate of 0.2 gram per plant). The fertilizer grade 15-15-15; N-P_2O_5-K_2O solution at the same

rate of urea were alternately added afterward. After 5 months, seedlings were transplanted to the plots. Seedlings were planted in the bottom of a furrow and soil was mounded over the plants as these develop to fill in and form raised bed. Bed size was 0.5x5 m, row spacing for seedling transplants was 1 m and seedling spacing was 0.5x0.5m. Plants obtained water irrigation regularly and moisture was maintained covering with coffee husks. For composted and non-composted cow manure treatments, the plants were applied with fertilizer grade 21-0-0; N-P_2O_5-K_2O at the rate of 30 kg/rai (or 15 grams per hill) after 10-15 days of transplanting. After one month, fertilizer grade 15-15-15; N-P_2O_5-K_2O was added at a rate similar to those of 21-0-0. Maintenance management of asparagus was weeding and insect control when necessary (Anon, 2010).

Kale

The cultivar large leaf Chinese kale seeds (Chai Tai, Bangkok, Thailand) was prepared by soaking them in the water for overnight before planting. For planting the seeds, about 5 seeds were placed at the bottom of planting pit and covered with soil. Spacing of rows and pits were 15x15 cm. Rice straws were slightly covered on the soil to maintain moisture level. After the seedlings had 4-6 mature leaves, they were thinned to one plant per hill. Water was regularly irrigated with sprinkler, and weed, and insect control were managed when necessary. Plants were fertilized with both composted, non-composted cow manures. Additional chemical fertilizer grade 12-8-8; N-P_2O_5-K_2O at a rate of 100 kg/rai was applied at 20^{th} day and 30^{th} day, respectively (Anon, 2010).

Coriander

Seeds of Coriander (Chai Tai, Bangkok, Thailand) were directly sown on the beds. The growing soil was covered with rice straws. Before sowing, the seeds were crushed to remove seed coat. Thereafter, they were overnight soaked in the water before planting. Seedling appeared in 10 to 20 days. After one month, fertilizer solution of 21-0-0; N-P_2O_5-K_2O at the rate of 15 g/20 L was sprayed for both composted cow manure and non-composted cow manure treatments in the quantity of 20 L/m^2. Weed and insect control were managed follow the recommendation of Anon (2010).

Stink weed

Stem was used for planting stink weed with spacing of 12x12 cm at the depth of 20 cm pit, before planting, its leaves were cut off approximately at 10 cm of the stem. Growing beds were covered with rice straws for moisture maintaining and camouflage with black netting plastic. It should be dimmed light at about 80%. After one month, the net plastic was removed so that the plants had obtained full sunlight. The plants were applied with cow manure and chemical fertilizer grade 20-20-0; N-P_2O_5-K_2O mixed with urea (46-0-0; N-P_2O_5-K_2O) at a rate of 50 kg/rai at 15 days after planting. Weeds and insect control were managed followed recommendation of Taweboeornkoul (2010).

Peppermint

Tip cutting in 3 to 6 inches-long young shoots were used for peppermint seedlings. They were grown in seedling trays with the same cultivating materials as asparagus growing. It was placed in green house and regularly irrigated using drip irrigation. After one month, transplanted the seedlings to the plots and camouflage net plastic black with 80% dimmed light. Spacing of row and hill was 10x10 cm. As with other types of vegetables, cow manure was applied additionally with chemical fertilizer grade 21-20-0; N-P_2O_5-K_2O at the rate of 10 kg/rai at 20-25 days after growing. Weeds and insect control were managed followed recommendation. After one month, black camouflage net plastic had removed (Anon, 2010).

3.3.4 Sample collection

Soil samples from each treatment plot were analyzed periodically at 7, 21, 35 and 49 days. Soil samples were randomly collected from between and around the plant stems. They were kept in plastic bags. Soil samples had thoroughly well mixed before analysis. They had to be immediately analyzed when the samples arrived in laboratory.

For vegetables, they were collected once at 49 days only. Asparagus were randomly collected from two beds per treatment by pulling shoot that emerged from the soil around 10 to 20 cm and they were kept in plastic bag. For kale, the plants were cut at the part beyond the soil. Whereas coriander and stink weed, their root, stem and leaves had to withdraw from the soil. Shoot of peppermint was collected by cutting at 12 cm long from its tip. All of the collected samples were immediately analyzed when arriving in laboratory without cleaning before examining.

3.3.5 Bacterial analyses

Samples of soil and vegetables were determined for contamination of *E. coli* and *Salmonella* spp. followed BAM method (Bacteriological Analytical Manual) (Feng *et al.*, 2002; Andrews and Hammack, 2007) the same as 3.1.1.1.

3.3.6 Measuring fresh and dry weight of vegetables

Each kind of vegetable had washed with water and rinsed, additive water was removed using tissue paper before weighing with balance (LIBROR EB-4300D, Shimazu, Japan). The vegetable samples were dried in the hot air oven at 75°C for 48 h. Dry weight of the vegetables were determined after cooling down in a dry environment.

3.3.7 Statistical analysis

Bacterial quantity had to convert into $\log_{10}$ count before statistical analysis. Statistic calculating program was satitistix 8.0 (Tallahassee, FL, USA). Data were analyzed by analysis of variance (ANOVA) using a split plot block design. The differences between the treatments were examined by Fishers least significant difference (LSD) test at a significance level of 95%.