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THESIS

RISK MANAGEMENT OF *ESCHERICHIA COLI* IN SWEET BASIL AND CORIANDER FOR EXPORT

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A Thesis Submitted in Partial Fulfillment of
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Pornpen Morakotjinda 2012: Risk Management of *Escherichia coli* in Sweet Basil and Coriander for Export. Doctor of Philosophy (Food Science), Major Field: Food Science, Department of Food Science and Technology. Thesis Advisor: Assistant Professor Warapa Mahakarnchanakul, Ph.D. 146 pages.

The objective of this research was to determine the sources of microbial contamination on exported sweet basil and coriander. Samples of investigation were farm environmental and utensils in packing house, practices during processing such as washing process were observed as well. The results showed that *E. coli* was detected in all environmental sources (seed, soil, fertilizer, and irrigation water) in sweet basil farm. While only soil in coriander farm was found *E. coli* contamination at 1.2-3.0 log CFU/g. The population of *E. coli* contamination in sweet basil farm environment were high in seed, soil and irrigation water with 4.8-4.9, 0.7-3.3, and 0.9-2.0 log CFU/g,ml, respectively. Surprisingly, low level of *E. coli* contamination was found in fertilizer (0.7 log CFU/g). According to the results from environmental swab samples from packing house (gloves, table, scissors and cover material), all observed utensils were in unhygienic condition and found *E. coli* contamination ranging from 0.2 to 4.9 log CFU/g,cm^2. The prevalence and population of *E. coli* contaminated in wash water taken from both farms were high (90-100%, 1.1-3.2 log CFU/ml). Microbiological results indicated that wash water could be the main source of cross contamination in packing house since the population of *E. coli* contaminated in vegetables after washing was higher than before washing. A total of 249 vegetable samples were collected throughout the fresh produce process from farm to factory in years 2009 to 2011, the trends of *E. coli* contamination in sweet basil and coriander increased throughout the process which ranged from 0-4.0 log CFU/g (year 2009) and 0-3.6 log CFU/g (year 2011). In 2009, 67% and 100% of exported sweet basil and coriander were contaminated with *E. coli*. However, the *E. coli* contamination in exported vegetables in year 2011 was less than year 2009. Further experiment was conducted to explore the possibility of cross contamination affected by 1) shaking force, 2) reused water, and 3) simultaneously washing with and without 50 ppm sodium hypochlorite. Results showed washing with 50 ppm sodium hypochlorite reduced *E. aerogenes* contaminated in vegetables by 1.1-1.4 log CFU/g, from the initial load 4.2 log CFU/g). No significant difference ($P\geq0.05$) when increasing washing time (from 5 to 15 min) and applying shaking force. While, washing with reused water, 9-46% of *E. aerogenes* in reused water was transferred to sound vegetables. The cross contamination between contaminated vegetables to wash water was inhibited when washing in chlorinated water. Cross contamination between contaminated and sound vegetable was inhibited when simultaneously washing in chlorinated water. Exposure assessment studies were conducted to describe which step of fresh produce process from harvesting to factory affected the population of *E. coli* contaminated in vegetables at receiving factory. Simple approach model and regression model were conducted. Sensitivity analysis was carried out by using tornado rank correlation and regression analysis feature in @Risk™ software. The population of *E. coli* contaminated in vegetables after harvesting step was the most factor influence the *E. coli* contamination at receiving factory. Then, risk estimation of illnesses caused by consuming the exported fresh produce contaminated with *Salmonella* spp. or *E. coli* were developed. The risk of illnesses per 100,000 populations when consume the sweet basil or coriander contaminated with *Salmonella* spp. were found to be 37.9 or 31.2 consumers from ingestion contaminated produce at 4.9 or 6.7 log MPN/serving, respectively. In case of enteropathogenic *E. coli* contaminated in sweet basil or coriander, 1.4×10^{-5} or 0 consumers become ill from ingestion of 0.03 or 0 log CFU/serving. The investigation of contaminated source in fresh produce process will assist to reduce the risk of *E. coli* contamination and develop the control strategies to enhance the food safety of Thai exported fresh produce.

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Student's signature Thesis Advisor's signature

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Pornpen Morakotjinda
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LIST OF ABBREVIATIONS

CFU/g	=	colony forming unit/gram
CFU/ml	=	colony forming unit/milliliter
CFU/cm^2	=	colony forming unit/square centimetre
°C	=	degree celcius
µl	=	microliter
cm	=	centimeter
g	=	gram
h	=	hour
l	=	liter
M	=	Meal size
ml	=	milliliter
ppm	=	part per million
P_E	=	Probability of exposure to pathogenic bacteria
P_i	=	Probability of illness
$P_i(D)$	=	Probablility of illness from dose: Beta Poisson
β	=	Beta-Poisson parameter
α	=	Beta-Poisson parameter

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RISK MANAGEMENT OF *ESCHERICHIA COLI* IN SWEET BASIL AND CORIANDER FOR EXPORT

INTRODUCTION

Fresh produce is one of the most significant exported agricultural products of Thailand. Tropical vegetables, herbs, and spices are commonly used as ingredients in many oriental food dishes and the number of Thai food restaurants in the western world hence there is a great potential market for these foods. Thailand has also established the national policy to become the kitchen of the world due to the rich natural resources and long agricultural experience. However, increasing production of fresh produce raises the question of whether or not farmers implement proper Good Agriculture Practices (GAPs) in their production, since the quality and safety of these agricultural commodities have to meet the requirements of the customer. There are many reports referring to the range of microorganisms associated with outbreaks linked to fresh vegetables, especially bacteria, particularly members of the *Enterobacteriaceae*, *Salmonella* spp. and *Escherichia coli* O157. Fruits and vegetables normally carry a non-pathogenic epiphytic microflora. However, many factors contribute to the microbiological contamination of these products with pathogens. Contamination can arise as a consequence of cultivation (soil, organic fertilizers and irrigation water), post-harvest handling (trimming, sorting and washing) and transportation (Beuchat 1996; Doyle and Erickson 2008; Taormina *et al*., 2009). Another aspect contributing to the microbial risk for the consumer is consuming the raw vegetables (European Commission, 2002).

During 2005-2006, the fresh produce exported from Thailand, including coriander and four varieties of basil, was detected as *Salmonella* spp. contaminated by the Health Protection Agency (HPA) at the point of entry (Border inspection post) in London and a pan-London retail (Zweifel and Stephan 2012; Johannessen and Cudjoe 2009). Moreover, in 2007, ready-to-eat fresh herbs (3,760 samples) were collected

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from retail premises in UK for determining the presence or absence of *Salmonella* spp. and level of *E. coli*. Among 15 of fresh herb exported from Thai and 4 were found contaminated with *E. coli* at a level higher than permissible limit (2 log CFU/g) (Elviss *et al.*, 2009). The incidence of pathogen contamination in this exported fresh produce affected fresh produce exports of Thailand. These commodities were banned and restricted, subjected to stringent microbiological inspection. In the beginning, the Thai government hurriedly conducted traceability in order to seek a root cause of this problem by inspecting Good Manufacturing Practices (GMPs) systems of export factories, as well as the production process, and GAP of the cultivating fields. However, it was still affecting to the credibility of Thai's exporters and reducing the values of the fresh produce exported at that time.

The microorganism decontamination by using antimicrobial agents in fruits and vegetables was wildly reported (Hana *et al.,* 2000; Keskinen *et al.,* 2009; Zhang *et al.,* 2009; López-Gálvez *et al.*, 2010). Various antimicrobial agents can be used to reduce the microbial load on fruits and vegetables. The most common antimicrobial agents used are chlorine-based compounds with free chlorine concentrations of 50-100 ppm (FAO/WHO, 2008), and chlorine compounds are still the common antimicrobial agents used in Thai factories. Therefore, this research was conducted to introduce chlorine compounds to prevent the contamination of pathogenic bacteria particularly during preparation in packing house and washing process.

The objective of this research was conducted to study 1) the microbiological quality of Thai exported fresh produce from farm to export in order to identify the source of contamination particularly *E. coli*, 2) to prevent the contamination between washing process, by using *Enterobacter aerogenes* as surrogate microorganisms during the washing process with and without sodium hypochlorite and 3) to estimate the risk from *E. coli* contamination in fresh produce using fresh sweet basil and coriander as the model.

Results from this study was expected to fulfill the understanding of the *E. coli* contamination in Thai fresh produce which identifies the potential source of

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pathogenic contamination in food chain from farm to packing house to find the core problem. In addition the data used as a model will be to develop the risk assessment system for other types of fresh produce from farm to fork. Moreover, the recommendation will assist to prevent and control the sources of contamination which will provide the valuable information of fresh produce of Thai exports. Then, the control strategies can be developed to prevent the contamination between its process in order to enhance the food safety of Thai exported fresh produce and increase the export potential in the future. The information of this research will enhance the food quality and safety of Thailand’s export commodities accepted, particularly fresh produce, to be trusted for customer and sustained in world market.

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OBJECTIVES

1. To investigate the prevalence of *Escherichia coli* contamination in farm and packing house environment and vegetables for export.

2. To identify the source of contamination of *E. coli* in order to prevent and control the contamination at primary production and packing house before transporting to factory.

3. To evaluate the cross contamination between washing process and the effect of chlorine on reduction of *Enterobacter aerogenes*.

4. To estimate the probability of illnesses when consuming the exported sweet basil and coriander.

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LITERATURE REVIEW

1. Fresh Produce

The consumption of fresh produce has increased from the past. Food habit today is changed since the consumers are concerned more about their healthy diet and benefits gained from healthy fresh vegetables. Therefore, there is growth in global trade of fresh produce and an expanding of the market for Thai fresh produce, increased from local to international markets. In addition, the convenience and the technology involved in transportation and logistics facilitated the market. The export produce from Thailand to Europe increased since the 1980s. After that, the European market is a major importing region of Thai tropical fruits and vegetables.

Generally, vegetables carry natural non-pathogenic and pathogenic microflora. During growth, harvest, transportation, and further processing including handling, the produce can be contaminated with pathogens from human or animal sources. The outbreaks of foodborne illness, particularly in consuming fresh produce, have been widely reported especially by *Salmonella* spp., *Shigella*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Campylobacter (*Elviss *et al*., 2009).

Since 2000, the export values of Thai fresh produce have not increased in line with its competitors from China, Vietnam, and the Philippines. In 2005-2006, the threat of pathogenic contamination in exported fresh produce had an effect on fresh produce export business, the values of exported fresh produce in 2005 was dramatically decreased with strictly inspection (Figure 1).

During 2005-2006, the facing of pathogenic contamination in exported fresh produce had an effect on fresh produce export business, the values of exported fresh had been dramatically decreased due to the loss of credibility in food safety issues.

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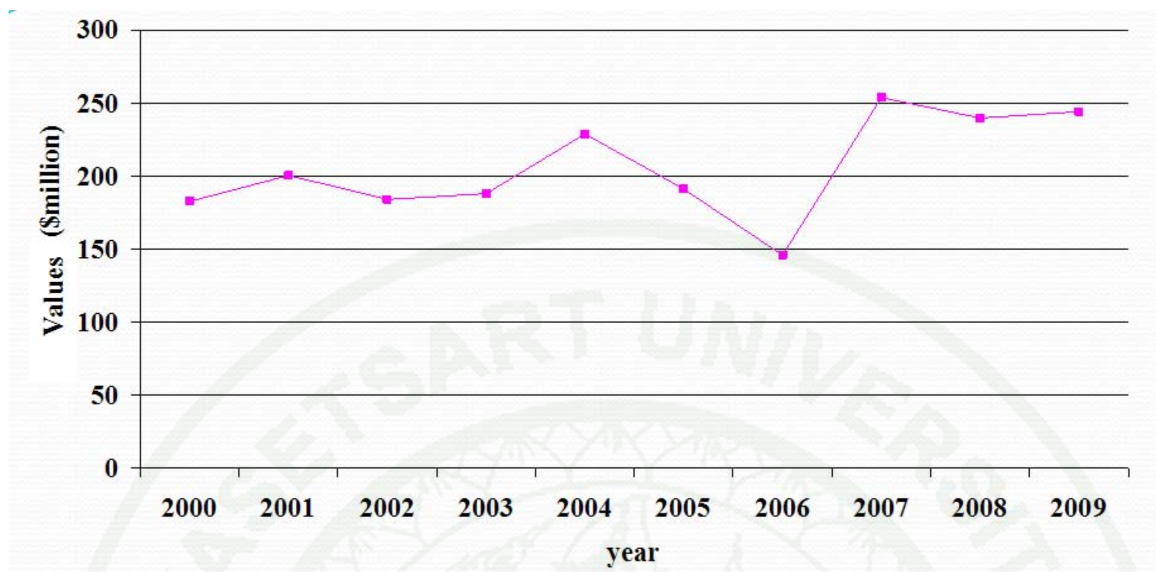

Figure 1 The export values of Thai exported fresh produce from 2000-2009

Source: FAOSTAT (2012)

In 2005, fresh herbs and spices (sweet basil, holy basil, paew leave, and mint) exported from Thailand were found to contain *Salmonella* spp. and *E. coli* (RASFF, 2005). Norwegian Scientific Committee for Food Safety (2008) reported that the Norwegian Food Safety Authority conducted the survey of 162 products, mostly from Thailand, and found that 28 % were contaminated with *Salmonella*, and 35 % with *E. coli* at > 100 CFU/gram. This resulted in a general prohibition of import of such products from Thailand. In the meantime, Finland had detected *Salmonella* spp. in 2 kinds of fresh produce leading to rejection and return of those products. Finland also reduced the credibility of fresh produce exported from Thailand. Moreover, the EU alert system urged the inspectors to seriously investigate the imported goods from Thailand especially in fresh cut and fresh produce (Matichon, 2005).

Recently, in 2011, 16 types of vegetables including varieties of basil, chili and capsicum pepper, eggplant, bitter gourd, and parsley were temporary banned after the European Union officials found some shipments contaminated with insects. Oversea consumer and Thai restaurants, especially those in Britain, had switched to order vegetables from Laos, Vietnam, the Netherlands and the Dominican Republic

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(Bangkokpost, 2011) which caused more or less the loss of Thai fresh produce marketing.

The main varieties of fresh produce which bacterial contamination problems were sweet basil (*Ocimum basilicum* Lamiaceae) and coriander (*Coriandrum sativum* Apiaceae). The quantity and export value of sweet basil and coriander were shown in Table 1. These vegetables are sold in supermarkets and used as ingredients in Thai food restaurants in western countries and some are used as garnish for western dishes.

Table 1 Quantity and value of sweet basil and coriander production in year 2006

Item	Quantity (kg)	Value (Baht)
Sweet basil	384,611	11,343,579
Coriander	536,638	17,648,222
Total	15,334,868	457,241,750

Source: Department of Agriculture (2006)

1.1 Overview of vegetable production and trade in Thailand

Mostly small production of vegetables in Thailand is supported by domestic consumption but some large production; for example asparagus, baby corn and okra, is intended for exporting. Vegetables are mostly grown in central and northern regions of Thailand such as Nakornpathom, Nonthaburi, Suphanburi, Ratchaburi and Chiang Mai.

Normally, Thai herbs and spices production comes from individual farm, which is different in agricultural and hygiene practices. While a contracted farm at which production size is larger, about 1.6 hectares, will produce large amount of fresh herbs and spices for export. Some producers are cooperative farms and have their own marketing. Usually contracted farms under the companies are given the primary

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agricultural needs (seed, pesticide) and the company will guarantee the vegetable's price. Therefore, contract farmers will gain the higher price compared to individual farmers, but they have to assure the quality of their product and implement systems such as GAPs. The traditional marketing chain in Thailand is characterized by many stakeholders. The modern trade is toward simplification, fewer steps and facilitate for transport, logistics, and cool-chain handling to maintain good quality for fresh produce (Figure 2). The local collectors in Figure 2 represent as packing house in our study. Packing house is part of the primary production; it functions as collected center, pre-preparation including pre-washing, distribution to local markets, wholesale markets, and factory for export.

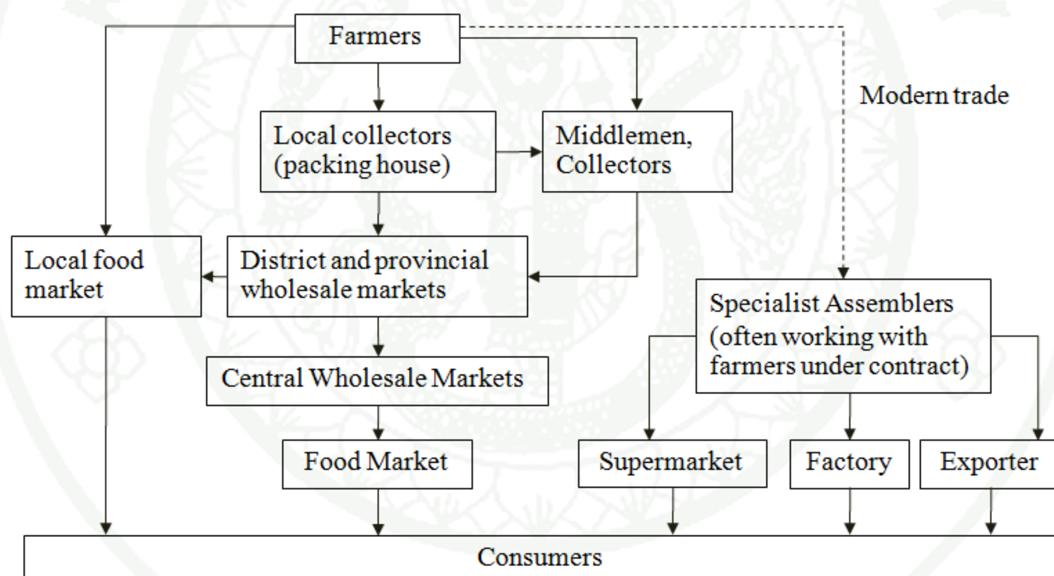

Figure 2 The distribution channels of vegetable trade in Thailand

Source: Adapted from Johnson *et al.* (2008)

In most cases, vegetables are generally washed after harvest by primary producers at farm or packing house, mostly with potable water. Washing with sanitizing agent to decontaminate the microbial load is widely applied in factory, but may not be common in farm or packing house. The hazards found in vegetables are not only microbial contamination but also pesticides, chemical fertilizers and

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herbicides. Vegetables could become contaminated with microbial pathogens by a wide variety of routes between farm environments with direct or indirect exposure via soil, manure, and irrigation water to vegetables. Moreover, the packing house and transportation might be the source of contamination, especially during the washing step at the packing house. The cross contamination may occur during preparing or trimming from utensil and worker at packing house, as well as in retail and food service establishments and in home kitchen (Winfield and Groisman 2003). Thus, risk of fresh produce consumption should be of increasing concern due to the possible of pathogen contamination from many sources.

1.2 Current situation in Thai's fresh produce

Currently, in 2010-2011, exported fresh produce from Thailand has been found pathogenic contamination by EU inspection (Table 2) (RASFF, 2011). Moreover, Thai fruits and vegetables exported to the EU have also been faced with insect contamination. Failure to meet EU standards may result in a suspension of some groups of vegetables, serious inspection that was taken could be used as a temporary self-imposed ban or a 100% check at port of departure. The major types of fresh produce which were in concern were as Thai aubergines, sweet basil, holy basil, coriander, mint, and some varieties of chili. Shortage of these ingredients cause trouble for many Thai businesses in the UK, the majority of which are made up of more than 1,500 restaurants and cafes, not to mention supermarkets and grocery suppliers (Amthaipaper, 2011).

Table 2 Contamination of pathogenic bacteria found in Thai fresh produce

Date	Types	Microorganism	Notifying countries
02-Jun-10	Mint	*S.* Weltevreden	The Netherlands
	Mint	*S.* Hvittingfoss	The Netherlands
	Coriander	*S.* Rissen	The Netherlands
	Cha om	*S.* Hadar	The Netherlands
	Cha plu leaf	*S.* Weltevreden	The Netherlands

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Table 2 (Continued)

Date	Types	Microorganism	Notifying countries
2-Jul-10	Cha phu leaf	*Salmonella* spp.[a]	The Netherlands
30-Jun-10	Sweet basil	*S.* Brunei	Finland
	Parsley	*S.* Augustenborg	Finland
5-Jul-10	Sweet basil	*Salmonella* spp.[a]	The Netherlands
30-Jul-10	Whole black pepper	*S.* Hvittingfoss	The United Kingdom
22-Dec-10	Mint	*Salmonella* spp.[a]	Finland
	Mint	*S.* Stanley	Denmark
Jan-11	Leaf vegetable and fresh spinach	*S.* Ndolo	Finland
	Leaf vegetable and fresh spinach	*S.* Bovismorbificans	Finland
	lemon grass	*S.* Wandsworth	Finland

[a]: Not identified serovar

Source: RASFF, 2011

***2. Salmonella* spp.**

Salmonella spp. is an infectious pathogen which occurs widespread in humans and animals throughout the world. They have been recognized as causes of enteric disease or known as salmonellosis for more than 130 years ago. *Salmonella* spp., the etiological agent of typhoid fever, was discovered by Eberth in 1880 and the organism was first cultured by Gaffky in 1884. In 1885, D.E. Salmon and T. Smith had isolated *Salmonella* Choleraesuis (previously written as *Salmonella choleraesuis*) from swine clinically diagnosed as having hog cholera and subsequently the genus *Salmonella* spp. was named in honor to D.E. Salmone's work in 1900. The first laboratory confirmed outbreak of forborne salmonellosis involved 57 persons who ate meat from sick cows. *Salmonella* Enteritidis was isolated from the organs of the victim who had

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not survived and from the meat and blood of the animal. Since then, *Salmonella* spp. has become a major cause of gastroenteritridis and enteric fever (Franz and Bruggen, 2008).

2.1 Pathogenesis of *Salmonella* spp.

Salmonella species are probably the most well-known bacterial foodborne pathogens. They are Gram negative, facultative anaerobic, rod-shaped, non-spore forming. They can metabolize a wide variety of organic substrates by both respiratory and fermentative pathways. Most *Salmonella* spp. are motile by peritrichous flagella and possess three major antigens: H or flagella antigen; O or somatic antigen; and Vi or capsular antigen. H antigen may occur in either or both of two forms, called phase 1 and phase 2. The organisms tend to change from one phase to the other. O antigens occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface. Vi antigen is a superficial antigen overlying the O antigen. *Salmonella* that have Vi-antigens are more virulent than others without vi-antigens such as *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin. Antigenic analysis of *Salmonella* spp. by using specific antisera offers clinical and epidemiological advantages. Determination of antigenic structure permits one to identify the organisms clinically and classify them to each serogroup. Presently 2,700 serovar was recorded and all of them are pathogenic bacteria (Franz and Bruggen, 2008).

Salmonella infections produce symptoms ranging from mild food poisoning (gastro-enteritis) to fatal typhoid fever. *Salmonella* spp. produces a haemolysin (called typholysin). Like other bacteria, *Salmonella* spp. has evolved the habit of invading host cells in order to hide from the immune system and to gain nutrients. In order to escape the gut into the surrounding tissues, *Salmonella* spp. invades the epithelial cells. Pathogenic *Salmonella* such as *S.* Typhimurium, have a pathogenicity that contains various toxins that mediate invasion and intra-cellular survival. The production of toxins also assists to avoid being phagocytosed by

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neutrophils and macrophages. Once inside the cell, *Salmonella* spp. resides in a compartment that does not fuse with lysosomes so that the bacteria are not exposed to proteases (Maciver, 2002).

3. *Escherichia coli*

Escherichia coli was isolated and described in 1895 by Dr. Theodore Escherich and subsequently, the organism was recognized as part of the flora of the intestinal tracts of warm-blooded animals and humans. *E. coli* is the predominant facultative anaerobe in the human bowel and recognized as parts of normal flora in the intestine. *Escherichia coli* is a genus of Gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacteria from the family Enterobacteriaceae. There are many *E. coli* strains that infect humans. Figure 3 shows the complex relationship of 6 pathotypes causing diarrhoeal disease. *E. coli* are the most numerous aerobic commensally inhabitants of the large intestine in humans. Pathogenic *E. coli* can cause a variety of diarrhoeal diseases in hosts due to the presence of specific colonization factors, virulence factors and pathogenicity associated genes. Six pathotypes are recognized. There are Verocytotoxigenic *E. coli* (VTEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC) and Diffusely adherent *E. coli* (DAEC) (O'Sullivan *et al*., 2007).

Verocytotoxigenic *E. coli* (VTEC)

E. coli can produce verocytotoxin (VT) or Shiga toxin (Stx). Cytotoxins production can disrupt protein synthesis within host cells. They have the ability to form attaching and effacing lesions (A/E lesions) on epithelial cells. These toxins are synonymously either called verocytotoxins (VT), because of their activity on Vero cells or Shiga toxins (Stx) similarity with the toxin produced by *Shigella dysenteriae*. Enterohaemorrhagic *E. coli* (EHEC) are a subset of VTEC. EHEC also possess an approximately 60-MDa (EHEC plasmid). *E. coli* O157:H7 is the most important

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EHEC serotype in relation to public health. The infectious dose of VTEC has been calculated to be as low as 10-100 cells.

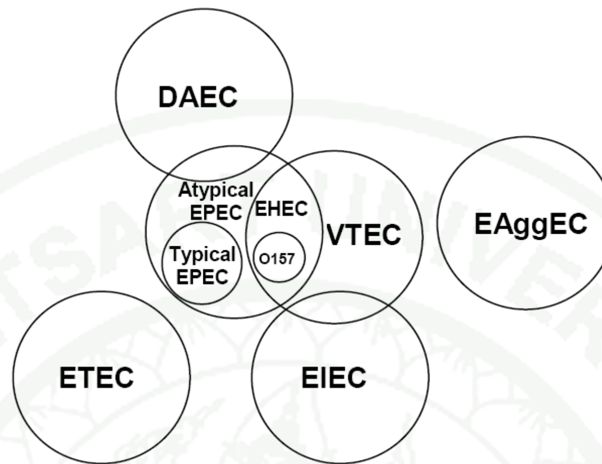

Figure 3 The relationship diagram of *E. coli* pathotypes causing diarrhoeal disease

Source: O'Sullivan *et al*. (2007)

Enteropathogenic *E. coli* (EPEC)

EPEC can adhere to the epithelial cells of the intestine, causing either watery or bloody diarrhea. This type does not produce any toxins.

Enterotoxigenic *E. coli* (ETEC)

They are a major cause of traveller’s diarrhoea worldwide. They can produce the heat-stable (ST) or the heat labile (LT) enterotoxins. These toxins cause inhibition of sodium absorption and stimulation of chloride secretion, which leads to watery diarrhea.

Enteroinvasive *E. coli* (EIEC)

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EIEC can invade the epithelial cells of the intestine resulting in a mild form of dysentery. EIEC strains have ability to invade into epithelial cells and disseminate from cell to cell.

Enteroaggregative *E. coli* (EAggEC)

EAggEC (or EAEC) are associated with acute or persistent diarrhoea, especially in developing countries. EAggEC strains are characterized by their ability to aggregatively adhere to tissue cells. They also produce an enteroaggregative heat-stable toxin.

Diffusely Adherent *E. coli* (DAEC)

DAEC are a major cause of urinary tract infections worldwide. They are identified by their adherence to Hep-2 cells in a diffuse pattern. DAEC are divided into two classes, those which harbour afimbrial adhesions (Afa)/Drori antigen (Dr) adhesions and those that express an adhesion involved in diffuse adherence, which is a potential cause of infantile diarrhea. DAEC infection is characterized by the growth of long finger-like cellular projections that wrap around the adherent bacteria.

4. Risk assessment

The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) are in the forefront of the development of risk based approaches for the management of public health hazards in food. Risk Analysis is a process consisting of three components: Risk Assessment, Risk Management, and Risk Communication, which has the overall objective to ensure public health protection (CODEX, 1999). The risk assessment part constitutes the scientific part of the risk analysis and embraces four elements: hazard identification, hazard characterization, exposure assessment, and risk characterization (Figure 4).

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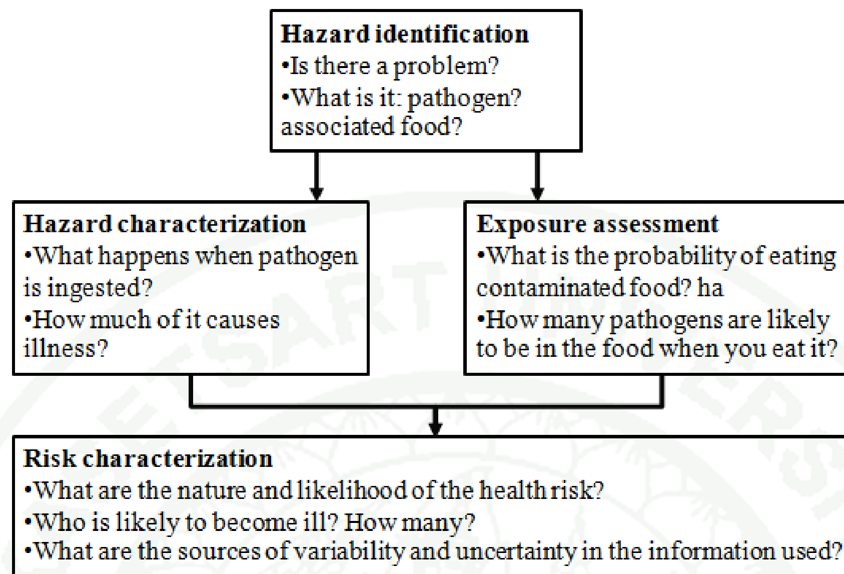

Figure 4 Steps and components in a microbiological risk assessment

Source: FAO/WHO (2002)

Microbiological risk assessment is an important tool to evaluate and explain the relationship between host, bacteria, and human. The CODEX Alimentarius Commission gives guidelines for the development of a microbial risk analysis and a set of principles and definitions. Moreover, Cassin *et al.* (1998a) introduced the process risk model (PRM) which integrates QMRA (Quantitative Microbial Risk Assessment) methodology with scenario analysis and predictive microbiology. PRM is a tool for identifying intervention strategies that might mitigate risk. Identification of hazards in food process with a systematic assessment could be useful for establishing food safety requirements for international trade.

Definition

Risk is a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food (European Commission, 2002).

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Hazard is a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect (European Commission, 2002).

Risk assessment for microbiological hazards in foods is defined by the CODEX Alimentarius Commission (CAC) as a scientifically based process consisting of four components which are hazard identification, exposure assessment, hazard characterization, and risk characterization.

The goal of a risk assessment may be to provide an estimate of the level of illness from a pathogen in a given population, but may also be limited to evaluation of one or several step(s) in a food production or processing system (FAO/WHO, 2003). When requesting a risk assessment, the risk manager should be specific with regard to the problem with which the risk manager needs to deal, the questions to be addressed by the risk assessment, and an indication of the measures the manager would consider or has available for the reduction of illness.

Hazard identification

During the past two decades, *Salmonella* Enteritidis has emerged as a leading cause of human infections in many countries, especially in vegetables eaten raw. (European Commission, 2002). Considering the different risk factors from farm to packing house would be useful to a risk manager in identifying the source of pathogen contamination that would have the greatest impact on reducing human infections. The hazard identification provides an in-depth review of available scientific data and information that identifies and qualitatively characterizes evidence regarding the sources of *Salmonella* spp. and *E. coli* infection arising from the consumption of exported fresh produce in the human population.

In Thailand, The National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health of Thailand was investigated *Salmonella* spp. and *Shigella* contamination in food and human (humans, foods raw material, ready-to-eat foods, frozen foods, frozen sea foods,

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animals, animal feeds, water, rectal swabs, stools, bloods, urines, and pus). The total number of *Salmonella* and *Shigella* and their identification was shown in Table 3. A few serotypes of Typhoidal *Salmonella* found in Thailand each year was reported in an annual report of confirmed *Salmonella* and *Shigella* in Thailand 2005-2008.

Table 3 Identification of *Salmonella* and *Shigella* srovars in Thailand (2005-2008)

Organisms	2005		2006		2008	
	Serovars	Isolates (%)	Serovars	Isolates (%)	Serovars	Isolates (%)
Non Typhoidal *Salmonella*	125	5270 (89.39)	131	3758 (89.54)	124	3491 (95.06)
Typhoidal *Salmonella*	2	35(0.59)	2	90(2.14)	2	6(0.16)
Shigella spp.	13	345(5.85)	11	180(4.29)	15	93(2.53)
Other bacterial	-	203(3.44)	-	151(3.60)	-	74(2.01)
No growth	-	43(0.73)	-	18(0.43)	-	8(0.21)
Total of samples	-	5896(100)	-	4197(100)	-	3666(100)

Source: National *Salmonella* and *Shigella* Center (2005-2008)

5. Important pathogenic bacteria in Fresh Produce

Fresh fruits and vegetables are increasingly recognized as a source of food poisoning (Lynch *et al.*, 2009). Among those food poisoning, *Salmonella* spp. and *E. coli* are mainly pathogenic bacteria of serious concern to public health. They are commonly found in natural environments and may contaminate food materials including fresh fruits and vegetables (Beuchat 1996; Thunberg *et al.*, 2002).

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5.1 *Salmonella* spp. and *E. coli* in fresh cut and fresh produce

Fresh produce is an important part of a healthy diet. During the last three decades, the number of outbreaks caused by foodborne pathogens associated with fresh produce consumption reported by the Centers for Disease Control and Prevention has increased. During 1973-1997, a total of 190 type of produce associated outbreak was reported and caused 16,058 illnesses, 598 hospitalizations, and 8 deaths. Produce-associated outbreaks accounted for an increasing proportion of all reported foodborne outbreaks with a known food item, rising from 0.7% in the 1970s to 6% in the 1990s. Foodborne outbreaks associated with fresh produce in the United States were increased in absolute numbers and as a proportion of all reported foodborne outbreaks (Sivapalasingam *et al.*, 2004).

Besides, the pathogenic organism such as *Salmonella* spp., *E. coli* O157:H7, *Shigella* spp., *Campylobacter jejuni* and *Listeria monocytogenes* was also associated in fresh cut and fresh produce. In 1970-2007, many varieties of fresh produce were found as contaminated items in many countries around the world (Table 4).

In 2006, the outbreak of acute gastroenteritis caused by enterotoxigenic *E. coli* (ETEC) serotypes O92:H and O153:H2 as well as *Salmonella* Anatum, which affected around 200 students and teachers after a high-school dinner in Greater Copenhagen, Denmark. Imported fresh basil used for preparation of the pesto was the most likely source of contamination (Pakalniskiene *et al*., 2009).

The microbiological criteria for *Salmonella* spp. and *E. coli* in ready-to-eat fresh herbs were established and used for international trading (Table 5). *E. coli* associated in herb could not be ≥100 CFU/g with free from *Salmonella* spp. (Elviss *et al.,* 2009).

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Table 4 Examples of fresh leafy vegetables and herbs reported to be associated with *Salmonella* spp. and *E. coli* (1970-2007)

Year	Product	Pathogen	Country
1993	Garden salad (carrots, iceberg, romaine lettuces, endive)	Enterotoxigenic *E. coli* O6:NM	Mexico and USA
1995	Lettuce leaf	*E. coli* O157:H7	USA (Montana)
1995	Lettuce leaf	*E. coli* O157:H7	USA (Maine)
1995	Lettuce leaf	*E. coli* O157:H7	USA (Illinois), USA (Connecticut)
1997	Lettuce, iceberg	*E. coli* O157:H7	Canada
1999	Lettuce	*E. coli* O157	Sweden
2000	Lettuce	*Salmonella* Typhimurium DF104	UK
2000	Lettuce, iceberg	*Salmonella* Typhimurium DT204b	England, Wales; Scotland; Iceland; Germany; the Netherlands
2001	Lettuce (in salad mix)	*Salmonella* Bovismorbificans	Australia (Qld)
2001	Salad pre-pack ready to eat	*Salmonella* Newport	UK
2003	Lettuce	*Salmonella* Braenderup	UK
2003	Lettuce	*E. coli* O157:H7	USA
2004	Lettuce	*Salmonella* Newport	UK
2004	Rucola/rocket	*Salmonella* Thompson	Norway (possible cases also in Sweden, England and Wales)

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Table 4 (Continued)

Year	Product	Pathogen	Country
2005	Lettuce (various mixes of romaine lettuce, red cabbage, carrots)	*E. coli* O157:H7	USA
2005	Lettuce, iceberg	*Salmonella* Typhumurium var Copenhagen DT104	Finland
2005	Parsley	*E. coli* O157:H7	USA
2006	Lettuce	*Salmonella* Typhumurium DT104	UK
2006	Lettuce, iceberg	*E. coli* O121:H19	USA
2006	Spinach	*E. coli* O157:H7	USA
2006	Basil	*Salmonella* Anatum, *enterotoxigenic E. coli* (ETEC)	Denmark
2007	Basil	*Salmonella* Senftenberg	UK, Netherlands, Denmark, USA

Source: Adapted from FAO/WHO (2008)

The causes of contamination of fresh produce process were identified as during cultivating, harvesting, production, transportation and consumption. The important sources of contamination were feces, manure, soils, water for cultivation, poor agricultural practices, and cross contamination during production process (Beuchat, 1996; European Commission, 2002).

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Table 5 Microbiological criteria for *Salmonella* spp. and *E. coli* in ready-to-eat fresh herbs

Food category	Microorganism	Microbiological quality (CFU/g)		
		Satisfactory	Acceptable	Unsatisfactory
Fresh herbs	*Salmonella* spp.	Not detected in 25 g	-	Detected in 25g
Pre-cut fresh herbs	*E. coli*	$\leq 10^2$	$>10^2$-$\leq 10^3$	$>10^3$
Ready-to-eat fresh herbs	*E. coli*	< 20	20-<10^2	$>10^2$

Source: Adapted from Elviss *et al.* (2009)

5.2 *Salmonella* spp. contaminated in fresh cut and fresh produces

In Thailand, the prevalence of *Salmonella* spp. contaminated in food was reported in 1993-1995. From the foodborne survey, *Salmonella* spp. was found in ready-to eat consumption ranging from 1.2% to 10%. The food vehicles associated with *Salmonella* spp. were in ready to eat food (3.48%), Som Tum (10%), salad (1.2%) and dessert (7.5%). The serotyping of *Salmonella* spp. associated in food was identified and 19 serova was reported, such as *S.* Derby (23.68%), *S.* Anatum (18.42%) (Rongrodejanarak *et al*., 1998).

Jungsamanukul *et al*. (2010) found the *Salmonella* spp. contamination in fresh produce that sold in local market and supermarket in Bangkok. The 7 samples from 80 samples of fresh produces (mint, lettuce, cabbage and basil) were found to have *Salmonella* spp. contamination.

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5.3 Prevalence of *Salmonella* spp. and *E. coli* in Thailand

In Thailand, Chaiyadat *et al*. (1999) studied the microbial load in ready to eat food. The samples were taken from the 25 shops in Department of Communicable Disease Control, Department of Health, Department of Medical Service, Office of the Permanent secretary and The Food and Drug administration (Thailand). Microbiological quality of ready to eat food tests were conducted according to the standard of microbiological quality of foods amended by Department of Medical Science. The results showed that 4.0% of samples had total bacterial count over the acceptable standard. In addition, 5.8% of samples were contaminated in excess of MPN *E. coli* limit.

Rimpranam (2000) also investigated the microbiological quality associated in fresh vegetable salad such as cucumber, lettuce, tomato, carrot, onion and corn. The results showed that the pathogenic bacteria such as *Bacillus cereus* (3.0-4.8 log CFU/g), coliform bacteria (2.2-6.1 log CFU/g), *E. coli* (4.5 log CFU/g), *Staphylococcus aureus* (3.3 log CFU/g) were detected. The amounts of total bacteria count and yeast/molds were detected around 3.2-7 and 4.8 log CFU/g, respectively. Later in 2010, ready to eat food sold in fresh-markets and by street vendors in Bangkok were taken to analyze for microbiological quality. Five types of the most favoured foods which are Khanomgeen-namya, Khanomgeen-namprik, Namprik-kapi, Namprikplaraa and Yum were taken were observed. Most of the food items were contaminated with *Escherichia coli* (48.3%) which had the highest amount of 7×10^4 CFU/g. In addition, *Bacillus cereus* (61.67%) with the highest amount 1.4×10^4 CFU/g, *Staphylococcus aureus* (5.00%) with the highest amount 2×10^3 CFU/g and *Clostridium perfringens* (25.83%) with the highest amount 4×10^3 CFU/g. Among 120 samples there were 7 samples contaminated with *Salmonella* (Mahakarnchanakul *et al*., 2010).

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5.4 *Salmonella* spp. and *E. coli* contaminated in exported fresh produce from Thailand

In Thailand, the concerned types of microorganism contamination in fresh produce are pathogenic bacteria group such as *E. coli, Salmonella* spp. and *S. aureus*. In 2005, Norway was reported that the fresh vegetables from Thailand were contaminated with *E. coli* and *Salmonella* spp. Moreover, in March, 2007, RASFF (Rapid Alert System for food and feed) of DG-SANCO reported that they found the *Salmonella* spp. contamination in frozen kefir lime leaves. Previously, in July-December 2006, RASFF warned about the food contamination for 16 items sold in EU markets and recalled the fresh produce exported from Thailand. From this evidence the conclusion can be reached that contamination of *E. coli* and *Salmonella* spp. was major problem for Thai fresh produce exported.

6. Factors affecting the pathogen contamination from farm to packing house

Soil

There are many risk factors which causing the pathogenic contamination in fresh produce production. Soil is a rich reservoir for a variety of microorganisms, it is not only the source of non-pathogenic flora but also pathogenic bacteria, such as *Bacillus cereus, Clostridium botulinum and Clostridium perfringens, Listeria monocytogene*. The normal flora is the main bacteria in soil and it impacts microbial ecology in soil (Gagliardi and Karns, 2002).

Pathogens present in manure leach through the soil profile at various rates depending on the soil type and management, rainfall and other factors (Beuchat, 2006). Pathogenic organisms from the human or animal reservoir can be found in soil due to irrigation and fertilization with un-composed manure and sludge or due to droppings of animals in the farming area (European Commission, 2002). Survival in the soil is influenced by many factors such as soil type, soil humidity and its variation of temperature and soil microflora (Islam *et al*., 2004; Ibekwe *et al*., 2011). The

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availability of organic constituents could be the main factor to limit the growth of bacteria (Garcia and McKay, 1970).

Fertilizer

The application of chemical fertilizers in agricultural production in Thailand has the tendency to increase in amount year by year. Many types of fertilizer using in agricultural field are chemical fertilizer, organic fertilizer, fresh manure, and composted manure. Many outbreaks in fresh produce may be caused by using of fresh manure and uncompleted composed manure. These fertilizers might promote the survival or proliferation of pathogenic bacteria in soil and vegetables and might contaminate to other sources (FAO/WHO, 2008).

Manure is a fecal matter which contains billions of microorganisms. There are many types of manure that are used in farming such as cattle, swine, poultry, and bat. The manure could be the source of pathogenic contamination. Pathogenic bacteria could not only survive for extended periods but also survive in manure-amended soils (Jiang *et al*., 2002) and may subsequently become associated in manure and cross contamination to vegetables (Islam *et al*., 2004). Generally, *Salmonella* spp. survives in manure longer than *E. coli* O157:H7. The survival time of *E. coli* O157:H7, *E. coli* O26 and *Salmonella* spp. are up to 6 months, 3 years and 2 years, respectively (Fremaux *et al*., 2007; Nicholson *et al.,* 2005).

Survival in manure is generally shortened with higher pH (Mukherjee *et al*., 2007), higher temperature (Himathongkham *et al,*. 1999; Semenov *et al.,* 2007)). Moreover, the fluctuation of temperature influences the survival of bacteria in manure. When the manure is applied soil, survival in manure-amended soil is generally reduced by higher temperatures, higher levels of native microflora, levels of easily available nutrients, increased levels of microbial diversity and lower clay content (FAO/WHO, 2008). *L. monocytogenes* was shown to grow in manure-amended soil when the inoculum density was low and reached higher levels in soils

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amended with solid chicken manure than in soils amended with either liquid hog manure or inorganic fertilizer (Dowe *et al*., 1997).

The important factors in reducing the bacteria during the composting process are the temperature and time. Then it is necessary to control the composting process by increasing the temperature to 50-60°C. To control the temperature during process, the piles system should be applied because it helps to maintain the temperature and moisture in the manure pile; moreover, it promotes the uniform composting process. This system should be maintained with the cored temperature of bio solids at 55°C or higher and stored for at least 1 month (Moon, 1997).

In general, increasing the delay time between the application of manure and harvest could reduce the occurrence of foodborne pathogens on fresh produce. During the composting process of manure, the microbiological process is not well understood. To prevent cross contamination, the composted manure should be applied at least 120 days before harvest (Tiquia, 2005).

Ontoum *et al*. (2010) investigated the temperature of stored manure and manure types (bat, swine, cattle, and chicken) affecting on the survival of *E. coli* and *Salmonella* stored for 21 days at 25 and 40°C. At 25°C, *E. coli* cells in bat manure and swine manure could survive for 12 days, while *E. coli* cells in cattle manure and chicken manure could survive shorter as 6 days. At 40°C, *E. coli* in bat manure survived for 6 days, but could not detect *E. coli* in swine manure, cattle manure and chicken manure since the first day of storage. Meanwhile, *Salmonella enteritica* serovar Hvittingfoss and Augustenborg in bat manure and swine manure survived for 21 days at 25°C and survived for 18 and 15 days in cattle manure and chicken manure, respectively. At 40°C, these two serovars survived in bat manure and cattle manure for 6 days, while cells could not be detected in swine and chicken manure since the third and first day of storage. Temperature and manure types affected on the survival of these two pathogens and provide information that exposing fertilizer to higher temperature (for example stored under sun-light) may assist to reduce *Salmonella* spp. and *E. coli* which may contaminate in those fertilizers.

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Irrigation water

Water is mainly used for irrigation of plants. There are many types of water source such as surface water, ground water, municipal potable, and non-potable water (Steele and Odumeru, 2004). Microbiological quality of water depends on its sources. Surface water in Thailand is categorized as poor or very poor quality while the microbiological quality of ground water is suitable for agriculture and livestock uses (Tirado *et al*., 2008).

Irrigation with contaminated water is a significant pathway for the contamination of vegetables with microorganism prior to harvest. Irrigation water can be a carrier of many microorganisms including pathogen strain of concern for human health such as *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella* spp., *Cryptosporidium parvum*, *Giardia lamblia*, *Cyclospora cayetanensis*, *Toxiplasma gondii* and hepatitis A viruses (Ferguson *et al*., 2003; Winfield and Groisman 2003; Aruscavage *et al*., 2006; Doyle and Erickson, 2008). In addition, the pathogens can associate with sediment (Loge *et al.,* 2002; Lu *et al.,* 2004). Therefore, the use of contaminated irrigation water can increase the frequency of pathogen isolation from harvested produce (Islam *et al*., 2004; Islam *et al.,* 2005; Barker-Reid *et al.,* 2009).

Plant surfaces can be contaminated by contact with contaminated irrigation or post-harvest wash water, soil, manure, wild animals, and human handling (Beuchat, 2006). Contaminated irrigation water which was exposed to the edible portion of vegetables may increase the microbial hazards, especially for those crops close to the time of harvest. Irrigation water can act as a vehicle for transport across a herd and into the watershed. The transfer of foodborne pathogenic microorganisms from irrigation water to vegetables depends on the irrigation technique and on the nature of the produce. Spray irrigation systems can increase the risk of contamination when compared with drip irrigation or flooding irrigation techniques (European Commission, 2002). Drip irrigation techniques could possibly be the potential system used in farming because the edible parts of most crops are not wetted directly and therefore decreases the risk of bacterial contamination from irrigation water and soil.

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Spreading of contaminated irrigation water on vegetables could be the problem. In September, 2006, *E. coli* O157:H7 outbreak associated with spinach affected over 200 people in 26 states in USA. The Center for Disease Control and Prevention (CDC) investigated how and why the spinach became contaminated. The environmental investigation of potential water issues related to the *E. coli* O157:H7 outbreak associated with fresh spinach was focused on irrigation water from 4 areas which were surface runoff from grazing areas onto cultivated fields, construction of irrigation wells, depths to groundwater, and groundwater-surface water interaction and direct use of surface water for irrigation. Of these factors, depths to groundwater and ground water-surface water interactions were the most likely water-related factors contributing to this outbreak, fecal samples from cattle and wild pigs and San Benito River water samples taken on Paicines Ranch matched the strain found in the patients and bagged spinach (CDHS-FDA, 2007). Moreover, Cooley *et al*. (2007) also reported that the surface water was a vehicle of transmission of *E. coli* O157 and a potential risk factor for pre-harvest contamination of leafy vegetables in the Salinas region of California.

Surface waters could contaminate with pathogenic bacteria (McGee *et al*., 2002). Surface water in Brisbane, Australia was collected to determine the prevalence and concentration of enterohaemorrhagic *E. coli* (EHEC) using quantitative PCR (qPCR) based methodologies. All samples (32 samples) were positive for *E. coli* which ranged from 1.0×10^{0}- 3.5×10^{4} CFU/100 ml. Moreover, 9 (28%), 14 (44%), and 15 (47%) were positive for EHEC O157 LPS, EHEC VT1, and EHEC VT2 genes, respectively (Ahmed *et al*., 2009).

The irrigation water quality and the selection of irrigation system should be awared to minimize the risk of harmful bacteria spreading to vegetables, the US acceptance criteria of irrigation water for edible plant parts dictates that fecal colifroms in 100 ml of water should be lower than 1,000 CFU. So far, this criteria have been in consideration as a long standing guidance in the US (United State Environmental Protection Agency, 2004).

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Microbial pathogens are known to survive for considerable periods of time in groundwater (John and Rose, 2005). Pathogenic bacteria such as *E. coli* O157:H7 can survive in irrigation and in farm water for 14 day at a temperature below15ºC. Moreover, addition of bovine feces (1% w/v) increased survival of *E. coli* O157:H7 up to 24 days (McGee *et al.,* 2002). *E. coli* can survive and multiply in irrigation water, wastewater, subtropical sediments, and mineral water (Islam *et al.,* 2005). The outbreaks of *E. coli* O157:H7 on spinach and processed lettuce in 2006 were investigated. From the investigation, the California Department of Health Services (CDHS) and U.S. Food and Drug Administration (FDA) identified contaminated irrigation water as a possible source of *E. coli* O157:H7.

Worker

Many pathogens can be transferred to the products by workers who are picking, packing, or handling the stuff (Schaffner and Schaffner, 2007). The failure of people working with food to wash their hands after using the toilet has been the cause of contamination and many foodborne illness outbreaks (Adams and Motarjemi, 1999). Good Hygienic practice of hand washing is the best solution to prevent cross contamination. The procedure was following from safe handling of fresh produce in the partnership for food safety education (McGlynn *et al*., 2009), the workers have to rub their hands together with soap for at least 20 seconds and clean under the nails and between the fingers, and also rub fingertips of each hand in suds on palm of opposite hand. The workers should wash their hands under clean and running water, and dry their hands with a single-use towel.

During work, the bacteria can contaminate from food surfaces to the hands of workers directly and via cutting board. To reduce pathogen contamination during cooking, using alcohol-based hand sanitizer can reduce the cross contamination (Schaffner and Schaffner, 2007). Hygiene and health of workers are very important factors for producing safe food. To reduce the cross contamination from worker to food, the education, hand washing facilities and award or bonus should be applied. In the field, the toilet facilities should be supplied with convenient and clean stuff. For

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hand washing, the liquid soap in dispensers, potable water, and single-use paper towels should be provided. The training program helps the workers in understanding the relationship between food safety and personal hygiene.

Wash water

Normally, post-harvest applications such as utensil cleaning and product rehydration and cooling, the water-quality criteria would be similar to that for potable water (drinking water; *E. coli*, less than 1 CFU/100 ml) (Stoeckel, 2009).

The washing process in the packing house needs to remove the soil and dust. Moreover, this step should reduce the microbial load in the vegetable. However this step should be recognized as the critical step to monitor the wash water (Gil *et al.*, 2009). Insufficient washing methods with contaminated water may be the source of microbial contamination when the insufficient washing method was applied.

A lot of researchers studied the cross contamination and sanitizer in the washing process (Allende *et al*., 2008; López-Gálvez *et al*., 2009). Maintaining the sufficient free chlorine concentration in wash water is critical for preventing pathogen cross contamination during the washing process (Luo *et al.,* 2011).

Bacteria transfer rate

Cross contamination is frequently proposed and referred to the transfer, direct or indirect, of microorganism from a contaminated product to a sound product. There are 3 specific transfer routes: Air-to-food (AF); Surface-to-food in fluids (SFF); and Surface-to-food by contact (SFC) (Pérez-Rodríguez *et al*., 2008). The AF occurs though dust particles or via aerosols especially during the preparation and packaging of products (Reij and Aantrekker, 2004). SFC transfer can occur in a wide variety of ways and situations such as through contact with contaminated food, utensils, cutting boards, and food handlers.

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The transfer data can be expressed by means of transfer rates (TR) (Chen *et al*., 2001), defined as the percentage of cells transferred from the donor surface to the recipient surface, which can be written as follows:

$$Transfer\ rate_{dr} = \frac{CFU_{recipient}}{CFU_{donor}} \times 100 \qquad (1)$$

where *Transfer rate* $_{dr}$ is transfer rate of bacteria from donor to recipient
CFU recipient is CFU on the recipient
CFU donor is CFU on the donor

It is important to note that the distribution of transfer rate is distinctly non-normal. Therefore log transfer rate is approximately normal distribution. To calculate the transfer rate, the log-transform of bacteria should be transformed (Schaffner, 2003). The transferability is strongly linked to bacterial attachment such as attachment force on surfaces. Factors affecting bacterial transfer can be divided into 2groups: 1) environmental and intrinsic factors, the physical-chemical properties of the surface material, moisture, pressure, and contact time, and 2) factors related to bacterial species such as the producing-capacity of exopolysaccharide, biofilm formation, clump formation and the presence of extra-cellular structures (curli and fimbriae). Several studies have reported that transferability is dependent on bacterial species and probably related to the difference in attachment characteristic between bacterial species (Midelet and Carpentier, 2002; Knobben *et al*., 2007).

Transfer rate is complicated by many factors and no one simple model can be easily used for all food products. The stochastic models (such as probability distributions) seem to provide a better fit to the apparent random and imprecise nature of the experimental data (Pérez-Rodríguez *et al*., 2008).

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7. Hazard characterization

The hazard characterization provides a description of the public health outcomes, pathogen characteristics, host characteristics, and food-related factors. The third major issue for dose-response modelers is variability associated with the host, with the pathogen and with the environment. The disease triangle relationship (Figure 5) from epidemiology literature suggests that the probability of illness is a very complex function to predict. The host must be susceptible to be attacked by the pathogen. The pathogen must be able to attack the host. The environment (food matrix) must favor the development of the pathogen. Disease is most severe when the environment favors pathogen growth and development over the host.

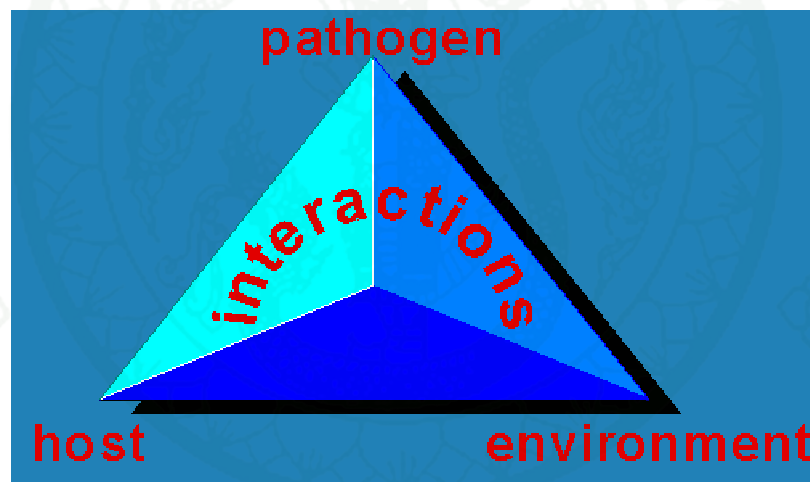

Figure 5 Disease triangle relationship

Source: Coleman (1998)

It also presents a review of information on relevant dose response models describing the mathematical relationship between an ingested dose of *Salmonella* spp and *E. coli* and the probability of human illness. An extensive review of available outbreak data was also conducted. From these data, a new dose-response model was derived using a publication of human feeding trial data about infective dose of

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E. coli O157 is estimated to be low (<10 viable cells) (Griffin and Tauxe, 1991) and a few hundred (Doyle *et a*l., 2001).

In several reports, Enteropathogenic bacteria, such as *Salmonella*, have been isolated from fresh herbs and leafy greens (Elviss *et al*., 2009; Zweifel *et al.*, 1995; McKee 1995). There have also been several outbreaks associated with the consumption of fresh herbs and leafy greens; e.g. an outbreak of *Salmonella* serotype Thompson associated with fresh cilantro in California (Campbell *et al*., 2001), outbreaks of *Shigella sonnei* and Enterotoxigenic *Escherichia coli* infections associated with fresh parsley in the United States and Canada (Naimi *et al*., 2003), an outbreak of *Salmonella* Senftenberg infection was linked to contamination of prepacked basil in England and Wales, 2007 (Pezzoli *et al.,* 2008). In 2006, there was an outbreak in Sweden associated with lime leaves used in a marinade. The lime leaves were imported from Thailand.

Incidence of Salmonellosis in Thailand

Example of *Salmonella* spp. outbreaks reported in Thailand from 1990-2005 caused by different kind of food (Table 6). However, no evidence of *Salmonella* spp. and *E. coli* outbreak via fresh produce in Thailand was reported.

Table 6 Incidence of Salmonellosis outbreaks in Thailand

Year	Cause	References
1990	*Salmonella* Enteritidis in chicken	Kantama and Jayanetra (1996)
31 October and 11 November 1999	Multidrug-resistant *Salmonella* Typhi in drinking unboiled spring water.in Poppra District, Tak Province	Swaddiwudhipong and Kanlayanaphotporn (2001)
August 5, 2005	*Shigella* and *Salmonella* spp out break in food from school in Bangkok, Thailand	Chanachai *et al.* (2008)

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Dose response

Dose response curves have been an important tool in microbial risk assessment (MRA). It links between the magnitude of exposure (dose) to a chemical, biological or physical agent and the severity and/or frequency of associated adverse health effects (response). Many researchers developed dose response modeling as a mathematical form for the relationship and those were fitted to the trial human feeding and foodborne illness outbreaks (Powell *et al*., 2000; Strachan *et al.,* 2005; Teunis, *et al*., 2010) (the parameter values shown in Table 7).

Typically dose-response models in microbiological risk assessment are in the concepts of non-threshold mechanisms (FAO/WHO, 2003). This results in the application of single-hit models like the Exponential model, Beta-Poisson, the Weibull-Gamma, and Hypergeometric model (Haas, 1983; Teunis and Havelaar, 2000) assume infection can occur only when a host has been exposed to (has ingested) one or more, each ingested cell acts independently, and all cells have the same probability of causing infection.

The data obtained from epidemiological and animal or human feeding frequently do not cover the entire dose response curve and are notoriously scattered. (Peleg *et al.*, 2011). The surrogate or mildly pathogenic bacteria were studied in human volunteers to estimate the dose response model. Surrogate bacteria could be either a pathogen with some similarity to the missing pathogen or a surrogate such as *Shigella* for *E. coli* O157:H7 or rabbits for *E. coli* O157:H7 (Crockett *et al.*, 1996; Haas *et al.*, 2000). The outbreak data can be used for setting the dose response model but it is often missing some data, such as exposed dose, etc (Teunis *et al*., 2008).

Beta-Poisson model

The Beta Poisson model (Haas 1983; Buchanan *et al*., 2000; Peleg *et al*., 2011) takes into account the variation that exists in pathogen-host interactions. The pathogen-host survival probability can be described by a probability distribution

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(Haas 1983). In this model, it is assumed that the dose is Poisson distributed and that one organism is sufficient to cause infection. It is the most common model which 2 parameters (Peleg *et al.,* 2011) as shown in Equation 2. The derivation of the approximation to the beta-Poisson model requires that β >> α.

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Table 7 The parameter values of dose response model for *Salmonella* spp. and *E. coli*

Pathogenic bacteria	Strain	Data	Model	α	β	σ	η	References
Salmonella spp.	*Shigella dysenteriae* M131	Human feeding trial	Beta-Poisson	0.2767	21.159	-	-	FAO/WHO (2002)
	Salmonella Enteritidis and Typhimurium	Outbreak	Single hit	0.0085	3.14	8.23	69	Teunis *et al*. (2010)
	Non-typhi *Salmonella*	Human feeding trial	Beta-Poisson	0.3126	2885	-	-	FAO/WHO (2002)
	Non-typhi *Salmonella* Naive	Human feeding trial	Beta-Poisson	0.4047	5587	-	-	FAO/WHO (2002)
		Outbreak	Beta-Poisson	0.1324	51.45	-	-	FAO/WHO (2002).
E. coli	STEC O157	Foodborne outbreaks of STEC O157 in children	Hypergeometric	0.084	1.44	-	-	Teunis *et al*. (2004)
	STEC O157	Foodborne outbreaks of STEC O157 in adults	Hypergeometric	0.05	1.001	-	-	Teunis *et al*. (2004)
	STEC O157	Rabbits feeding	Beta-Poisson	0.49	190000	-	-	Haas *et al.* (2000)
	EPEC/*Shigella*	Human feeding trial	Beta-Poisson	0.221	3110000	-	-	FSIS (2001)
	Shigella dysentariae and *Shigella jlexneri*	Human feeding trial	Beta-Poisson	0.162	15.86	-	-	Crockett *et al.* (1996)

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$$P_{\inf}(dose) = 1 - \left(1 + \frac{d}{\beta}\right)^{-\alpha} \qquad (2)$$

where $P_{\inf}(dose)$ is the probability of infection after ingesting

d is the number of pathogen units (dose, CFU)

α is model (infectivity) parameter

β is model (shape) parameter

The relationship of dose to response can be illustrated as a graph called a dose response curve. In this study, the dose response curve (Figure 6-7) for *Salmonella* spp. and *E. coli* from FAO/WHO (2002) and FSIS (2001) were used. Both models were estimated by using trial feeding data of human feeding trial of non-typhi *Salmonella* Naïve and surrogate pathogens EPEC/*Shigella*.

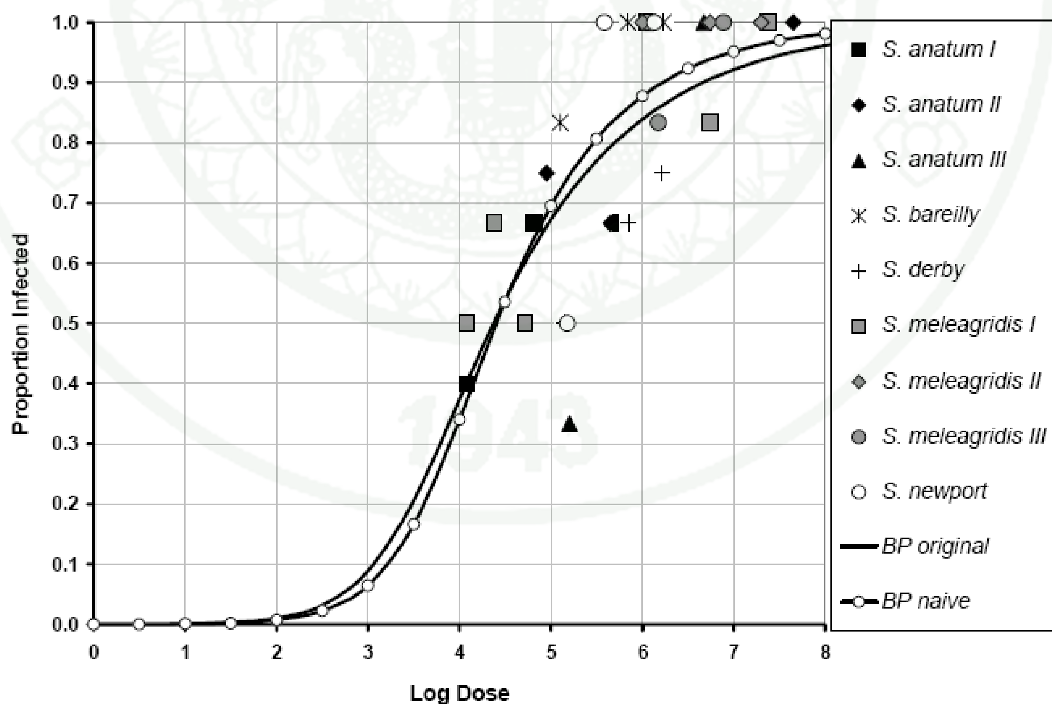

Figure 6 Dose response curve for non-typhi *Salmonella* naïve

Source: FAO/WHO (2002)

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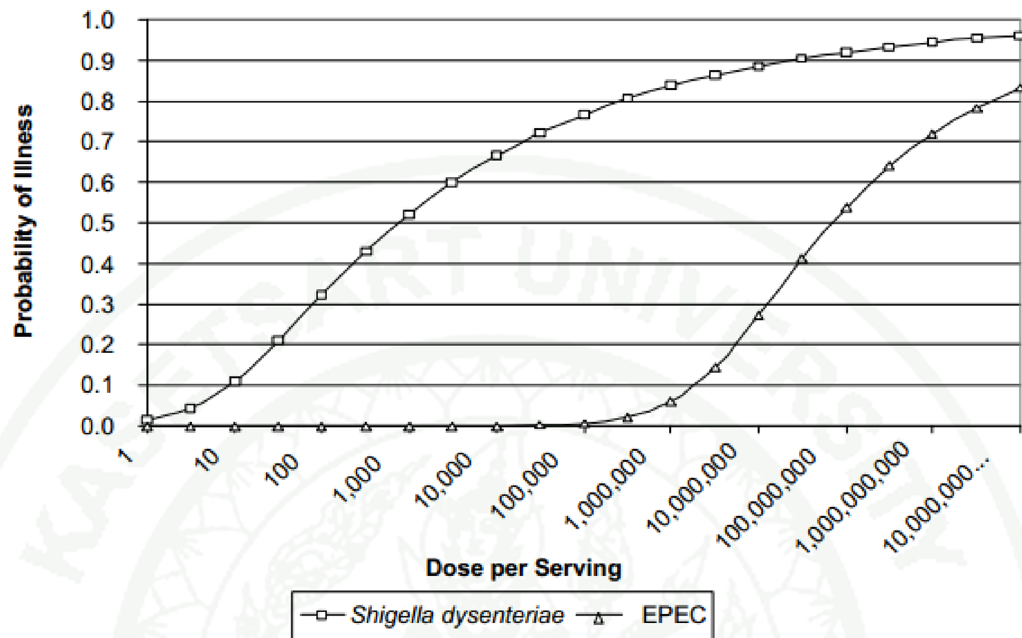

Figure 7 Dose response curves for enteropathogenic *E. coli* (EPEC)

Source: FSIS (2001)

Exponential model

The exponential model (Haas, 1983) is the simplest dose response model. If organisms are ingested from a dose ‘‘*d*’’, and organisms survive for at least one to cause infection, then the probability of infection (P_{inf}) can be expressed as:

$$P_{inf}(\text{dose}) = 1 - \exp(-rd) \quad (3)$$

where $P_{inf}(\text{dose})$ is the probability of infection after ingesting

d is the number of pathogen units (dose, CFU)

r is model parameter specific for pathogen

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8. Exposure assessment

Exposure assessment evaluates the likelihood that a person will be exposed to a hazard. The purpose of this step is to describe the consequences of exposure to a pathogen as an estimate of the magnitude of adverse effects for an individual consumer or a population. This step also provides the measure for evaluating the value of food safety efforts; e.g. a decrease in the number of people becoming ill and/or the severity of illnesses as a result of an intervention. This step should estimate prevalence and concentration of *Salmonella* spp. and *E. coli* contamination of exported fresh produce at the time of consumption and the amount of the product consumed at each meal. Then, in this research, the occurrence and number of *Salmonella* spp. and *E. coli* that may be presented in exported fresh produce and resulting products that contribute to the dose ingested by the consumer we are considering.

9. Risk characterization

CODEX defines the risk characterization step as the process of determining the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment. The probability of illness is derived by combining the number of organisms ingested (from the exposure assessment) with information on the dose-response relationship (hazard characterization).

Risk assessment will also help the industry to develop more effective HACCP plans. For the future, risk assessments will help plants to scientifically develop HACCP plans. For instance, the food processing industry can use a risk assessment to help identify hazards that are reasonably likely to occur. The best information which food plants may have now is qualitative-for example, whether a hazard presents a high, medium, or low risk. The real benefit to this change is that a hazard would be

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defined in terms of the risk of an adverse human health consequence, rather than in terms of contamination of the food.

Risk assessment also plays an important role in international trade by ensuring that countries establish food safety requirements that are scientifically sound and by providing a means for determining equivalent levels of public health protection between countries. Without a systematic assessment of risk assessment, countries may set requirements that are not related to food safety and could create artificial barriers to trade. Recognizing the importance of this science-based approach to fair trade, the World Trade Organization requires each country's food safety measures to be based on risk assessment.

As a first step for setting the risk assessment, it is often necessary to start the modeling downstream of consumption and estimate the changes that occur to the hazard as it progresses along the harvest to consumption chain. In addition, if the goal of the assessment is to detail the pathways leading to exposure so that the impact of the various elements in the pathway can be quantified in terms of their contribution to the overall risk to human health, then a Process Risk Modeling (PRM) approach (Cassin *et al.*, 1998a,b) can be used.

The process risk model approach is simply a modular approach to model a complex system. In the case of food system, the approach has been to separate the system into modules that logically and sequentially progress in a similar order to food system itself. This research focuses on the fresh produce exported from farm to packing house and agricultural field. The starting point and specific modules of this system were classified and determined by the assessor. A schematic representation from harvest-to-packing house and field model was shown in Figure 8.

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For the exposure assessment of this study, this research focuses on the fresh produce exported before entering factory or at factory receiving the raw material, the investigation starts from farm, harvesting, packing house, transportation until the vegetables arrive to the factory.

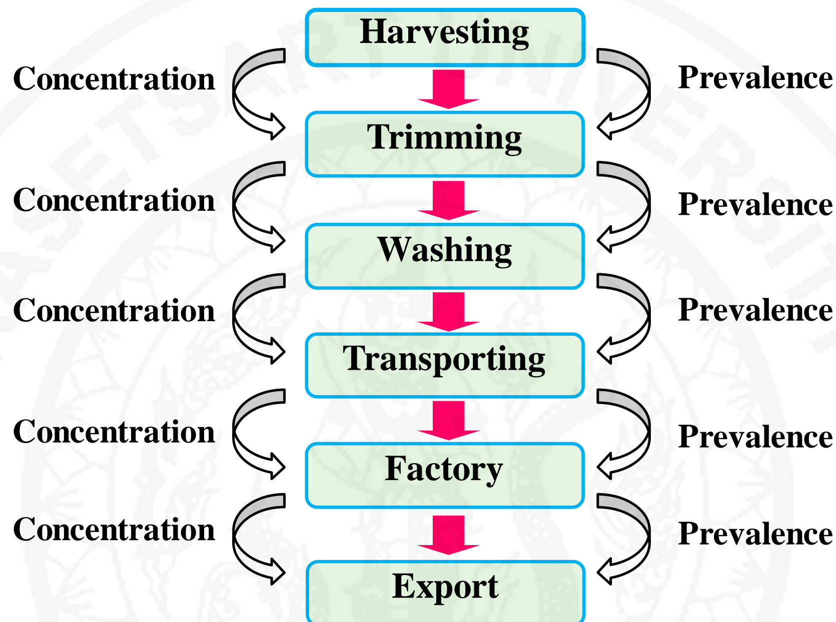

Figure 8 Process risk model of exported vegetables

In this study, the process risk model principle will be applied when the simple approach model was conducted for fresh produce production from farm to packing house. The population of *E. coli* contamination on vegetables after harvesting, after trimming, after washing, and after transportation (receiving at factory) were analyzed to identify which step will impact on the population of *E. coli* contamination on fresh produce when it arrives at the factory. The estimated risk upon consumption of exported fresh produce contaminated with *Salmonella* spp. and *E. coli* required the 2 parameters which are prevalence (P) and concentration (C) of *Salmonella* spp. and *E. coli* at consumption time (exported fresh produce).

In order to access the risk of sweet basil and coriander, the simple approach model (modified from Rodriguez *et al*., 2011; Danyluk and Schaffner 2011) and

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regression model (Osiriphun *et al*., 2011) were conducted to investigate the risk factors in process risk model (Figure 8).

10. Sensitivity analysis

Sensitivity analysis is an examination of the impact of changes in a model's input parameters on the solution. This method is a screening analysis to identify the most important input to propagate through a model in probabilistic framework. It helps to distinguish exposure pathways that are not important parameters but it cannot be used to prove that a particular exposure pathway is important (Cullen and Frey, 1999).

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MATERIALS AND METHODS

Materials

1. Hypothesis

During the years 2009-2011, the manure fertilizer is expected to be the main source of *E. coli* contamination in the fresh produce production from farm to packing house. From observation, we found the pathogenic bacteria in vegetables may spreader out during the washing process by washing vegetables in used water leading to the cross contamination between unclean and sound vegetables.

2. Materials

2.1 Culture Media

2.1.1 Bufferred peptone water (Merck Laboratories, Darmstadt, Germany)

2.1.2 Fluorocult *E. coli* O157:H7 agar (Merck Laboratories, Darmstadt, Germany)

2.1.3 Lauryl sulfate broth (Merck Laboratories, Darmstadt, Germany)

2.1.4 MacConkey agar (Difco Laboratories, Michigan, USA)

2.1.5 0.1% Peptone water (Merck Laboratories, Darmstadt, Germany)

2.1.6 Petrifilm aerobic count plates (3M Microbiology, Minnesota, USA)

2.1.7 Petrifilm *E.coli*/coliform count plates (3M Microbiology, Minnesota, USA)

2.1.8 Petrifilm yeast and mold count plates (3M Microbiology, Minnesota, USA)

2.1.9 Phosphate buffered saline (0.1 M, pH 7.2) (Fisher Scientfic, Pennsylvania, USA)

2.1.10 Tryptic soy broth (Difco Laboratories, Michigan, USA)

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2.2 Chemical Agents

2.2.1 0.1 M Hydrochloric acid (HCl) (Fisher Scientfic, Pennsylvania, USA)

2.2.2 Iodine-sodium thiosulfate redox titration (Oxidizer kit 322, Ecolab, Minnesota, USA)

2.2.3 Nalidixic acid (Sigma Chemical Co., Missouri, USA)

2.2.4 Sodium hypochlorite (NaOCl) (Fisher Scientfic, Pennsylvania, USA)

2.2.5 Sodium thiosulphate ($Na_2S_2O_3$) (Fisher Scientific, Pennsylvania, USA)

2.3 Equipments

2.3.1 Autoclave (Hirayama model HA-300 MII, Japan)

2.3.2 Centrifugation (Micro 7: Fisher Scientific, Pennsylvania, USA)

2.3.3 Digital balance (Ohaus model GT 4100, Japan)

2.3.4 Hot air oven (Memert model SLM600, Germany)

2.3.5 Incubator (Memmert model 700 D 06063, Germany)

2.3.6 Laminar flow cabinet (Gelman Sciences, Australia)

2.3.7 Micropipette (Gilson, USA)

2.3.8 pH meter (Jenco electronics model 6071, China)

2.3.9 Stomacher (Seward stomacher, Lab blender, England)

2.3.10 Timer (Heatthrow Sciences, USA)

2.3.11 Vortex mixer (Vortex genie-2 model G-560E, USA)

2.3.12 Glassware

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3. Laboratory

3.1 S.A.P. laboratory Co., Ltd

S.A.P. laboratory's services were used for *E. coli* identification in this study and this company actively participated in WHO Global Salm Sury External Quality Assurance System 2007 (WHO-EQAS program).

Methods

The prevalence and population of *Escherichia coli* were assessed to evaluate the risk of consuming the exported sweet basil and coriander. This study investigated the level of *E. coli* contamination in farm environments (seed, soil, fertilizer, and irrigation water), environmental sample and utensils using in packing house (food handlers' hands or gloves, table, scissors, wash water, cover material, and baskets) and vegetables collected from various steps throughout the production, from farm to packing house, as well as the vegetable samples for export. Moreover, the cross contamination pattern during washing process was conducted to understand its contamination behavior in order to suggest the control measures to be implemented in packing houses. The information of prevalence and concentration of *E. coli* was conducted in this research. The prevalence and concentration of *Salmonella* spp. provided by the previous study of Ontoum (2010) entitled "Contamination of *Salmonella* spp. on exported fresh produce during primary production".

Thus, the establishment of the exposure assessment of *Salmonella spp.* and *Escherichia coli* in exported fresh produce in this study was done using mainly results from this study and data from Ontoum (2010). Secondary data such as the prevalence, epidemiology data and consumption data relating to the outbreaks of *Salmonella spp.* and *E. coli*, were obtained from literature review.

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1. Microbiological qualities of environmental samples and fresh produce throughout the production process from farm to packing house of Thai exported fresh produce

According to the history of *Salmonella* spp. and *E. coli* contamination in fresh produce in 2009, the factories facing the problem were chosen to represent. Two types of exported fresh produces which have been contaminated were selected as the models, sweet basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) from six selected farm in Nakornpathom province. Sweet basil samples were collected during 2 harvesting periods, March 2009 from farm SA and May 2011 from farms SB and SC. Coriander was collected during July 2009 from farm CA and June 2011 from farms CB and CC. These six farms were different in weather and environmental conditions during growing and farm management. Environmental samples were collected from SA and CA, vegetables samples were collected from eight different locations. All farms and packing houses were located in Nakornpathom. Two factories were located in Nakornpathom (SA and CA) and the other two factories were located in Bangkok (SD and CD). A total of 466 samples were studied. Farm code, number of samples, and sampling plan were described as follows (Tables 8-9).

Table 8 Farm code and number of samples collected

Sample	Farm	Sweet basil		Coriander	
		Code	No. of samples	Code	No. of samples
Environmental samples	A	SA	91	CA	96
Vegetables collected from packing house	A	SA	20	CA	25
	B	SB	30	CB	20
	C	SC	30	CC	20
Vegetables collected at factory	A	SA	25	CA	25
	D	SD	42	CD	42
Total			**238**		**228**

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1.1 Sample collection

Samples from sweet basil and coriander farm environment (seed, soil, fertilizer, and irrigation water), utensils (hands/gloves, table, wash water, cover material, and basket), vegetable (after harvesting, after trimming, after soil removing, after washing, after transportation (at receiving factory), throughout the process from farm to packing house, were collected to enumerate for aerobic plate count, yeast and mold count, and *E. coli*. Number of samples is shown in Table 9.

Table 9 Number of environmntal samples and vegetable samples collected from farm and packing house during pre- and post-harvest

Source	Sample	No. of Samples collected		
		Sweet basil	Coriander	Total
Farm (Pre-harvest)	Seed	3	3	6
	Soil	15	15	30
	Fertilizer	15	15	30
	Irrigation water	15	15	30
Packing house (Post-harvest)	Gloves	10	NA	10
	Bare hands	NA	10	10
	Table	10	10	20
	Scissors	10	NA	10
	Cover material	5	8	13
	Basket	5	10	15
Water (Post-harvest)	Wash water	3	10	13
Vegetables (Post-harvest)	After harvesting	20	20	40
	After trimming	30	30	60
	After soil removing	NA	5	5
	After washing	30	10	40
	After transportation (at receiving factory)	31	31	62
	Exported fresh produce	36	36	72
	Total	**238**	**228**	**466**

NA: Not analyzed

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1.1.1 Seed

Twenty five grams of sweet basil (3 samples) and coriander seed (3 samples) growing on the farm were collected into PE bags (5 plots per bag). The sweet basil seed was produced by the farmer but the coriander seed was a commercial brand. The samples were transported to laboratory within 6 hours and then analyzed immediately.

1.1.2 Soil

Soil samples were taken during March-July, 2009. Samples included soil during preparation (before cultivation) and during plant cultivation. Thirty soil samples from sweet basil farm (15 samples) and coriander farm (15 samples) were collected in different locations. Each location represents as a plot (✕) shown in Figure 9. Thus, ten single plots (100 g/plot, 10-15 cm depth) were taken to collect as one sample under aseptic techniques. The collected samples were transported to laboratory within 6 hours and then analyzed immediately.

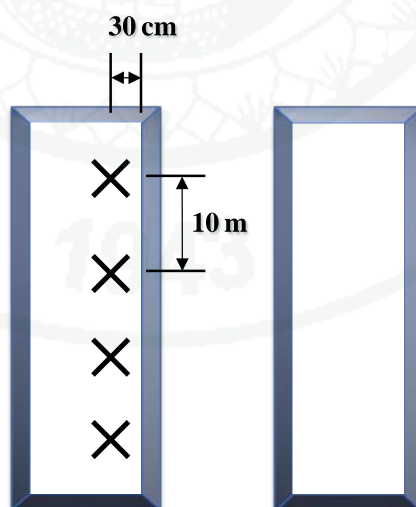

Figure 9 Sample collection of farm soil

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4.1.1.3 Fertilizer

Samples of 30 composed manure (150-250 g/sample), applied in the sweet basil (15 samples) or coriander farm (15 samples), were collected from 3 plots per stack within the 0, 15 and 30 cm depth by aseptic techniques. The samples were collected in plastic bags (PE), transported to laboratory within 6 hours after collecting and then analyzed immediately.

4.1.1.4 Irrigation water

Two types of irrigation systems used in farms were different in sources and management. Overhead irrigation system (with spray devices) and ground water was applied in coriander farm while the furrow system was supplied in sweet basil farm. Irrigation water samples from sweet basil (15 samples) and coriander farm (15 samples) were analyzed. Thirty samples, each 250 ml/ sample, were taken (10-20 cm depth within 10 m apart) into sterile duran bottle under aseptic techniques, kept in insulated box (4-7°C), transported to the laboratory in 6 hours and then analyzed immediately.

4.1.1.5 Gloves/Bare hand

Samples of 10 gloves or 10 bare hands were taken from workers handling vegetables at packing house. Random samples from the left hand (palms) during trimming were swabbed. Area of each hand sample (2x5 cm) was swabbed as shown in Figure 10. Swab samples were taken before starting work (2 samples), during working (6 samples) and after working (2 samples). Gloves samples were collected, put in the sterilized plastic bag, and transported to laboratory within 6 hours after collecting. Ten glove samples were taken in a similar procedure at before, during and after trimming. At the laboratory, the area of 10 cm^2 of each glove was cut by sterile scissors and then analyzed for microbiological quality.

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Figure 10 Sampling area of gloves taken for microbiological analysis

4.1.1.6 Scissors

Both sides of scissor blade (Figure 11) used for trimming sweet basil leaves and stems were collected before starting, during, and after working by swab method under aseptic techniques. The collected samples were kept in an insulated box (4-7°C) and transported to laboratory within 6 hours after collected. None of these was collected from coriander farm because workers used their bare hand to remove coriander leaves and stems instead of using scissors during preparation.

4.1.1.7 Table

During trimming, vegetables were placed on stainless steel sheet cover table. Randomly 10 area samples (2x5 cm) were swabbed before working, during working and after working under aseptic techniques. The collected samples were kept in insulated box (4-7°C), transported to laboratory within 6 hours after collected and then analyzed immediately.

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Figure 11 Sampling area of scissors taken for microbiological analysis

4.1.1.8 Washing water

Thirteen wash water samples, each 100 ml, from sweet basil packing house (3 samples) and coriander packing house (10 samples), were randomly collected during 2 hours of washing process. The collected samples were taken in duran bottle 250 m, kept in an insulated box (4-7°C), transported to laboratory within 6 hours and then analyzed immediately.

4.1.1.9 Cover material

To avoid the quality loss of vegetable particularly prevent wilt and weight loss, farmers usually use wet cloths to cover these fresh vegetables. Ten cm^{2} of cover material sample (5 samples for sweet basil, 8 samples for coriander) were randomly cut and taken to analyze the presence of bacterial contaminants. All samples were shipped to the laboratory within 6 hours and then analyzed immediately.

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4.1.1.10 Basket

After washing and draining the excess water, washed vegetables were packed into plastic baskets (60×40×40 cm). Samples of 15 baskets, sweet basil (5 samples) or coriander (10 samples), were randomly collected. Swab samples were taken from 3 areas per basket (top, middle, and bottom of basket) to obtain the total area 10 cm^2. All samples were kept in an insulated box (4-7°C) and shipped to the laboratory within 6 hours and then analyzed immediately.

4.1.1.11 Vegetables

Throughout the process, vegetables from 6 processing steps (after harvesting, after trimming, after soil removing, after washing, at receiving factory, and exported fresh produce) were collected. Randomly sampling at least 3 spots from each vegetable pile to obtain the total of 400-600 g were represented as one sample. These samples were placed in plastic bag (PE) and kept in an insulated box. The samples were transported to laboratory and analyzed within 6 hours. At the laboratory, each sample (whole vegetable) was randomly cut into pieces about 2x2 cm, mixed, then 25 g of each sample was weighed and analyzed for microbiological quality.

1.2 Microbiological analysis

1.2.1 Enumeration of aerobic plate count, yeast and mold and *E. coli*

Twenty five grams of each sample was homogenized for 2 min in 225 ml of buffer peptone water using stomacher at high speed. The samples were serial diluted with 0.1% peptone water. To determine total aerobic bacteria count, *E. coli* count, and Yeast and Mold count, one ml of serial dilution (10^{-1}-10^{-6}) was plated to petrifilm aerobic count plate, petrifilm *E. coli* count plates (EC plate), petrifilm Yeast and Mold count plate in duplicate, respectively. The *E. coli* and

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aerobic count plates were incubated at 37°C 48 hr. Yeast and mold plates were incubated at ambient temperature (30°C) for 72-120 hr.

The AOAC International and U.S. FDA Bacteriological Analytical Manual (BAM) define coliforms as gram -negative rods which produce acid and gas from lactose during metabolic fermentation. Coliform colonies growing on the petrifilm EC plate show darker red. Gas trapped around darker red coliform colonies indicates confirmed coliforms. Petrifilm EC plates contain violet red bile (VRB) with an indicator of glucuronidase activity. Most *E. coli* produce beta-glucuronidase which produces a blue precipitate associated with the colony. About 95% of *E. coli* produce gas, indicated by blue to red-blue colonies associated with entrapped gas on the petrifilm EC plate, these colonies were counted and reported as generic *E. coli*. But *E. coli* O157 was not specifically detected by using petrifilm EC plate.

1.2.2 Enumeration of *E. coli* O157:H7

During May-June, 2011 the 184 vegetable samples were obtained from four farms and two factories in Thailand. These samples included: vegetables after harvesting (sweet basil 20 samples, coriander 20 samples), after trimming (sweet basil 20 samples, coriander 20 samples), after washing (sweet basil 20 samples), after transport to factory (at receiving factory) (sweet basil 21 samples, coriander 21 samples) and exported fresh produce (sweet basil 21 samples, coriander 21 samples). These samples were transported to the laboratory in an insulated box, and bacteriological examination was undertaken immediately.

To avoid chilling injury on fresh vegetables, ice cubes were placed on the bottom of an insulated box (4-7°C) then covered with clothes to prevent ice directly contacting the samples. Insulated box used in this study has size about 60×100×60 cm.

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1.2.3 Sample preparation for *E. coli* isolation

A 25-g portion of each sample was homogenized for 2 min in a homogenizer stomacher with 225 ml of lauryl sulfate broth. One ml of mixed broth was plated on EC plate. The mixed broth was incubated at 35°C for 24 hrs aerobically. One loopfull of enriched broth was stick on fluorocult *E. coli* O157:H7 agar in triplicate and the plates were incubated at 35°C for 24 h. The expected *E. coli* O157:H7 colony (*E. coli* 0157:H7 is sorbitol-negative strains. It does not lead to any change in the color of the culture medium and thus proliferate as greenish colonies. *E. coli* 0157:H7 is not capable of forming β-D-glucoronidase. When irradiated with long-wave UV light, no fluorescence is formed) was purified. *E. coli* O157:H7 was confirmed using serological test (*E. coli* O antiserum O157 and *E. coli* H antiserum H7) by SAP laboratory (certificated under WHO-EQAS program).

2. To evaluate the cross contamination between washing process and the effect of chlorine on reduction of *Enterobacter aerogenes*.

2.1 Bacteria strains and inoculum preparation

Enterobacter aerogenes cells (*Enterobacter aerogenes* B199A, a nonpathogenic microorganism resistant to nalidixic acid) were grown overnight at 37°C in tryptic soy broth containing 50 µg/ml. nalidixic acid. Cells were harvested by centrifugation at 5,000 × g for 3.5 min and washed three times in phosphate buffered saline (0.1 M PBS, pH 7.2). Cell pellets were resuspended in phosphate buffered saline. The initial concentration of stock solution is ~10^8 CFU/ml. Appropriate 10 fold dilutions in phosphate buffered saline were made to determine the cell density of the inoculum and enumerate samples. Then, 0.1 ml of two lowest dilutions was plated in duplicate on MacConkey agar containing 50 µg/ml nalidixic acid. Spread plating was done in duplicate. Agar plates were incubated at 37°C for 24 h prior to enumeration.

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2.2 Natural *Enterobacter aerogenes* in vegetables

For detecting *E. aerogenes*, fresh basil and coriander were obtained from a supermarket. Damaged leaves and stems were removed using a sharp knife. Representative 25 gram samples each items were tested to confirm the absence of the test strain by added 225 ml of peptone water and homogenized for 1 min in a stomacher. Serially diluted technique was applied and spread plated onto MacConkey agar containing 50 µg/ml nalidixic acid. Agar plates were incubated at 37°C for 24 h.

2.3 Basil and coriander inoculation

Fresh basil or coriander was completely submerging into the bacteria suspension (~4.5 log CFU/ml for basil and ~5 log CFU/ ml for coriander) and shaking by manual for 5 min at ambient temperature (25°C). After inoculation, the samples were dried in a biosafety cabinet for 30 min. Inoculated air-dried samples were stored in a sterilized bag at 4°C for 24 hrs before use. The appropriated level of *E. aerogenes* on basil and coriander was 4 log CFU/g. The inoculated bacteria in samples were enumerated in duplicate.

2.4 Wash solution preparation

Wash solutions containing 0 (clean tap water) and 50 ppm of sodium hypochlorite (NaOCl) were prepared. Solutions with 50 ppm available chlorine were prepared by adding the appropriate volume of a concentrated solution of sodium hypochlorite (NaOCl) to deionized water. The pH of the sodium hypochlorite solution was adjusted to 6.8 using 0.1 M hydrochloric acid (HCl). Washing solutions were prepared one day before application. The initial free chlorine concentration was measured before used by using test kit (oxidizer kit).

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3. To evaluate the effect of washing method on *E. aerogenes* reduction

Ten grams of contaminated basil and coriander were washed by completely submerging into the clean tap water (90 ml, 25°C), compared between with and without manual shaking for 0 (no submerge in wash water, control), 5, 10 or 15 min. Then the contaminated vegetables and wash water were collected. Then contaminated vegetables were mixed with 90 ml of peptone water for 1 min. Then vegetable samples and wash water were serially diluted in peptone water and plated onto MacConkey agar containing 50 µg/ml nalidixic acid. Agar plates were incubated at 37°C for 24 h.

Similar procedure was done when washing contaminated vegetables with chlorinated water. After washing vegetables in chlorinated water, the vegetables were immediately neutralized by adding 0.05% sterile sodium thiosulphate ($Na_2S_2O_3$) (by 1:5 w/v) for stopping any residual bacteriostatic or bactericidal activity for 1 min. Then 40 ml of peptone water was added. The sample of chlorinated water was also neutralized by adding 0.1 ml of 0.05% sterile sodium thiosulphate ($Na_2S_2O_3$) immediately and free chlorine concentration was determined. To measure the free chlorine in wash water, the chlorinated water was taken at 0, 5, 10 and 15 min and immediately measured by using an iodine-sodium thiosulfate redox titration kit.

4. To evaluate the effect of reduced water on *E. aerogenes* contamination during washing process

First washing: Each 10 gram of contaminated basil and coriander was washed by submerging into tap water (90 ml) at 25°C for 5 min. After 5 min, the wash water and the contaminated basil or coriander was taken out and the *E. aerogenes* count was determined. The rest of the wash water, considered as reused water, was used for further washing.

Second washing: In order to simulate the reused water in washing process, 10 gram samples of sound (cleaned) basil or coriander were washed by submerging into the reused water (wash water from first washing) for 5 min. The wash water and the

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contaminated basil or corianders were taken out for microbiological analyses. The rest of wash water was used for the further washing.

Third and forth washing: Ten gram samples of sound basil or coriander were washed by submerging into the reused water (wash water from previous washing) for 5 min. Similar procedure was done as the previous washing and considered as the third and forth washing, respectively.

The population of *Enterobacter aerogenes* contaminated in all samples was analyzed to determine whether cross contamination occurred during washing with reused water.

5. To evaluate the cross contamination while simultaneously washing contaminated and sound vegetables

Ten gram of contaminated and sound vegetable were placed into each side of the same stomacher bag and simultaneously washed by the clean tap water (200 ml, 25°C), without manual shaking, for 0 (control), 5, 10 or 15 min. Then the contaminated and sound vegetables were taken out of the wash water. The samples were mixed with 90 ml of peptone water for 1 min. Then, serially dilution was done. The wash water were serially diluted in the similar procedure by adding peptone water and then diluents was plated onto MacConkey agar containing 50 µg/ml nalidixic acid. Agar plates were incubated at 37°C for 24 h. Similar procedures in contaminated and sound coriander were conducted and analyzed for *E. aerogenes.*

6. Data analysis

Data (obtained from washing experiment) was analyzed at $p<0.05$ for significant differences by ANOVA and Duncan's multiple range tests (Statistical Analysis System). Data were expressed as means ± standard deviation of three replicates or depending on the experiment. To estimate the probability of illnesses when consuming the exported sweet basil and coriander, the simulation of the model

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and the probability distribution of data were performed by using @RISK (Palisade Corporation; Ithaca, NY) for 10,000 iterations for each simulation.

7. Place and Duration

This research was conducted at the Department of Food Science and Technology, Faculty of Agro industry, Kasetsart University (Thailand) (during 2009 and 2011) and Rutgers University (USA) (year 2010). The sampling sites in Nakornpathom and Bangkok included 2 farms, 6 packing houses, and 4 factories. All samples were obtained between March, 2009 and July, 2011.

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RESULTS AND DISCUSSION

1. Field study

After interviewing export companies, one sweet basil and one coriander farm located in Nakornpathom province were chosen in this research based on companies' recommendation. Both farms produce vegetables supply for both domestic and export; their farm management followed Thai GAPs.

The purpose of the field study was to observe the practices and the overall sanitation at sweet basil and coriander farms and their packing houses. Samples of interest were also taken to evaluate the prevalence of *E. coli*, and *Salmonella* spp. (Master thesis of Ontoum, 2010). The total plate count and yeast and mold count were evaluated as well.

1.1 Farm environment

Among sweet basil and coriander farms, we found some of them had hygienic practices as shown in Figure 12. The facilities, such as toilet, were located in the farm area, thus there was the possibility that sewage water from the toilet could be released and contaminated into furrow using as the irrigation water. Fish being raised in the pond furrow were found which we considered as a contamination source.

Generally, the Good Agricultural Practices (GAPs) suggests that pound water used for irrigation of plants should be of good quality. Animals should keep away from the pond. The direct runoff form cultivation areas and swage water should not get into the pond. Toilets should be provided for the farm workers and should not be closed to sources or in places where rain can wash out contaminants or cause spills.

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Figure 12 Practices in the farm environment (Farm SA)

According to the observations from the farm premises, there was a poultry farm located near the sweet basil farm and it was possible that it was a source of pathogenic contamination; the run-off of waste from the poultry farm to vegetable plantation during heavy rain for example. Besides, the harvest practices on the farm are commonly done by bare hand. Bare feet were also observed among workers in preparation (Figure 13).

The fertilizer was basically stored in the open area which is shown in Figure 14. There are many types of fertilizer applying in sweet basil and coriander farms such as chemical fertilizer, composted, and pasteurized manure. Composted hog manure was commonly used on the sweet basil farm, the others were chicken and bat composted manure. Some were a mixture of hog and chicken manure. While composted manure use on the coriander farm was done at field site by mixing composted hog, chicken, and bat, then the mixed composted manure sent to be pelletized later (at different site). None of these farms used the green or un-composted manure. The farmers followed GAPs recommendation that manure should be fully composted before applied into land.

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Figure 13 Practices of workers in the farm environment (Farm SA)

Figure 14 Fertilizer storage in open area (Farm SA and CA)

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1.2 Packing house environment

Trimming area

At the packing house, the trimming area was located beside a farm which basically was the living house of the farm owner. In theory, the trimming tables should be cleaned before and after process. Since farmers have not been trained what different between hygienic and unhygienic cleaning, the trimming tables were cleaned with poor hygienic practices using broom to remove dirt before washing out with tap water. However, trimming table was covered with stainless steel sheet which will be easy to clean and disinfect. Moreover, since the area was the housing zone, we observed no barrier to prevent pets (dogs) from getting into this area which was considered as a source of contamination (Figure 15).

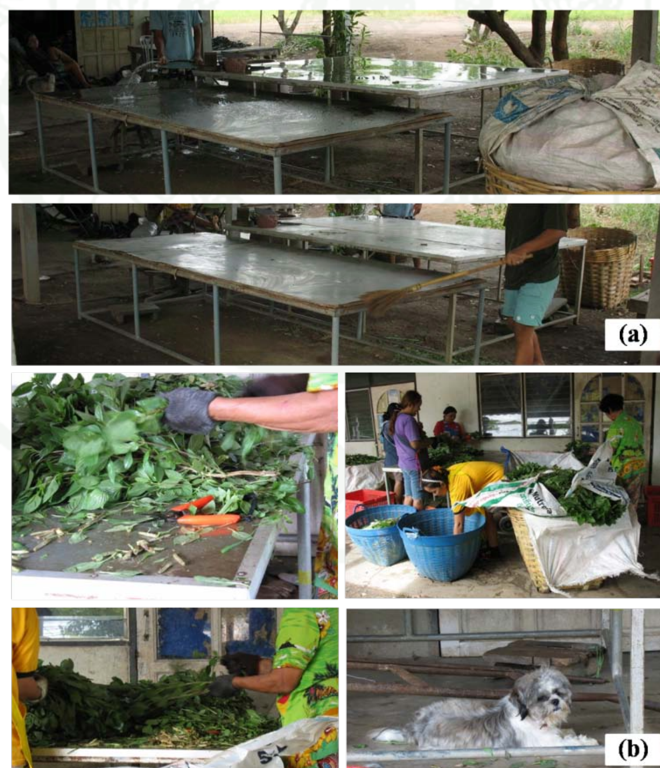

Figure 15 The hygienic practice on sweet basil preparation in packing house at farm SA, cleaning tables (a), and during trimming (b)

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1.3 Washing step

The purpose of washing at the packing house was mainly to increase vegetable's weight and prolong freshness; the added benefit of this is to clean. Adding sanitizer in wash water is considered as part of the cost of production. While a factory provides chlorine compound to one of these observed farms, the improper concentration of compounds was used due to lack of understanding and training (Figure 16). From our observation, the vegetables were washed in repeat water without adding sanitizing agent. The soil and dust contaminated in vegetables were removed from vegetables and they hanged on the wash water. The wash water becomes extremely dirty to build up of organic matter and bacteria. We noticed that this washing step might be the main source of contamination. It was not only increasing the pathogenic bacteria by washing process, but also spreading out pathogenic bacteria to clean vegetables.

1.4 Container

After washing, the vegetables were collected in the container. Normally, the bamboo or plastic baskets (40×60×40 cm, HDPE plastic basket) were used (Figure 17). Bamboo baskets are commonly used on Thai farms and they are not easy to clean, the plastic basket has been adopted instead and is used widely. The baskets should be cleaned with sanitizing agent and tap water. In this observation, the baskets used in both packing houses (sweet basil and coriander) were cleaned with tap water or washing water but without disinfectant agent. The GAPs recommends that the container should be washed and disinfected regularly before and after used. The packing containers should be raised on pallet to avoid contamination.

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Figure 16 Wash water using in washing step (Farm CA)

Figure 17 Plastic basket using in packing house (Farm SA)

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1.5 Cover materials

During transportation, the cover materials such as blankets (Figure 18a) or banana leaves (Figure 18b), were applied for protecting the vegetables from water loss. If the moist blanket has not been cleaned then it might be a possible source of contamination. We observed that the blanket was dipped into washing water to moisten it in order to keep vegetables cool and prevent water loss, the contamination from the washing water to vegetables via dirty blanket could occur.

Figure 18 Cover material for the fresh produce during transportation (Farm SA(a) and CA(b))

2. Prevalence of *E. coli* contamination in fresh produce from farm to packing house

According to our field study, the environmental samples from farm (seed, soil, fertilizer, and irrigation water), utensil samples (gloves, table, scissors, cover material) and wash water from packing house taken from farm SA and CA in Nakornpathom province were evaluated for the prevalence of *E. coli*.

At the sweet basil farms, *E. coli* was detected in each environmental sample (Table 10). The prevalence and population of *E.coli* were high in seed at 4.81-4.86 log CFU/g, moderate level of contamination was found in soil (0.70-3.31 log CFU/g)

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and low level of contamination was found in fertilizer (0.70 log CFU/g). Irrigation water was found positive *E. coli* sample as 60% with ranging from 0.18-2.01 log CFU/ml (Table 10). However, the positive samples found in irrigation water were low at >0-<10 CFU/ml (33.33%) (Table 11).

At the coriander farms, the population and prevalence of *E. coli* in soil samples taken from coriander farm were quite high ranging from 1.17-3.00 log CFU/g (66.67%) (Table 12), although 47% of samples were lower than 10 log CFU/g (Table 13). It appeared that soil could be the source of contamination among the pre-harvest samples, since the levels of contamination in seed, fertilizer and irrigation water were not detected *E. coli* (detection limit was 5 cfu/g) compared to similar sample type taken from sweet basil farm.

According to our observated information, we expected that irrigation water used on the sweet basil farms could be contaminated via waste water from toilet located in farm. Moreover, *E. coli* has a possible chance to get into water reservoirs from attached animal farms (duck farms located near sweet basil farm). Fresh water close to livestock farming systems may therefore represent a potential reservoir for enteric pathogens, increasing the potential for the organism to spread (Czajkowska *et al.,* 2005; McGee *et al*., 2002).

The prevalence and concentration of *E. coli* contaminated in irrigation water depend on the source of water. Duffy *et al*. (2005) reported that *E. coli* was detected in all types of irrigation water sources (cement canals, dirt canals, furrow, reservoir, river and well), though with low amount of *E. coli* (0-1.7 log CFU/ml). Geldreich and Bordner (1971) reported that 100% (10/10) of irrigation water samples obtained from well were contaminated with *E. coli* at mean concentration of 0.7 log CFU/ml, while 75% (15/20) of water collected from furrow system was contaminated with *E. coli* (0-1.1 log CFU/ml). Irrigation water collected from cement canals were contaminated with *E. coli* 0.1 log CFU/ml (3/50), which demonstrated that water-soil interface of the irrigation canal can be a reservoir for fecal population transported in the channel. On this observation, 2 types of irrigation systems used in farms were different in

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sources and management. Overhead irrigation system (with spray devices) and ground water was applied in coriander farm while the furrow system was supplied in sweet basil farm.

The method and water source of irrigation plays an important role in the contamination of fresh produce. Therefore the use of other methods like drip irrigation in lettuce production can considerably reduce the risk of crops contamination (Barker-Reid *et al*., 2009). Irrigation methods that subject the plant to direct contact the contaminated water increase the risk of contamination (National Advisory Committee on Microbiological Criteria for Foods, 1999).

We had assumed that composted manure used as fertilizer in sweet basil farm was the source of contamination; however the prevalence and concentration of *E. coli* in fertilizer showed very low at 0.7 log CFU/g (Table 10). In addition, fertilizer used on coriander farm (Table 13) was not detected to contain *E. coli* (detection limit was 5 cfu/g) because all have been completely composed.

Composed process could reduce the number of pathogenic bacteria contaminated in manure. Forshell and Ekesbo (1993) reported that the survival of *Salmonella* in cold cattle manure was longer than in composted cattle manure. *Salmonella* Dublin, *Salmonella* Senftenberg and *Salmonella* Typhimurium survived less than 7 days in composted cattle manure. But in cold cattle manures, *Salmonella* Dublin survived 183 days while *Salmonella* Senftenberg and *Salmonella* Typhimurium survived 204 days. In composted sow manure, *Salmonella* Senftenberg and *Salmonella* Typhimurium survived less than seven days while *Salmonella* Derby survived 14 days.

Nicholson *et al*. (2005) found that *E. coli* O157, *Salmonella* spp. and *Campylobacter* spp. survived in dairy cattle slurries and dirty water for up to three months at <20°C. In contrast, all these pathogens survived for less than one month in solid manure heaps where temperatures greater than 55°C were obtained. Results obtained from Jiang and coworker (2003) also supported that the large populations

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(10^4 to 10^7 CFU/g) of *E coli* O157:H7 survived for 36 days during composting in a bioreactor at an external temperature of 21°C but were inactivated to undetectable levels after 7 to 14 days when the external temperature of the bioreactor was 50°C. Solid manure storage for 1 month is probably sufficient to ensure elimination of most pathogens, at least 55°C within the main body of manure stack (Nicholson *et al*., 2000).

From our survey, the fully composted fertilizer was applied in both the sweet basil and coriander farms. The pathogen could not be detected in fertilizer. Unfortunately, 1 sample collected from the fertilizer used in a sweet basil farm was contaminated with *E. coli*. It might have been contaminated during handling and storage at the farm.

The persistence of fecal bacteria in the soil has been reported. Jones (1999) described *E. coli* that survived for at least 60 days in soil at 25ºC and for at least 100 days at 4ºC. Bolton *et al*. (1999) detected *E. coli* O157:H7 in soil 99 days after a fecal suspension containing this organism was applied to grassland. In the study by Ingham *et al*. (2004), when no manure was applied, bird and/ or mammal recontamination was the cause of the apparent persistence of indigenous *E. coli* in manure-fertilized soils. In addition, the persistence of *S.* Typhimurium in soil amended with inoculated poultry manure, compost, cattle manure compost and irrigation water has been studied (Islam *et al*., 2004). The pathogen remained viable on soil for 203-231 days. Survival was the greatest in soil amended with inoculated poultry manure compost. The pathogen was detected on radishes and carrots 84 and 203 days, respectively after seeding.

The results of *E. coli* count on seed (sweet basil) shows that the quality of seed is important to food safety aspect. It could be the source of contamination and pathogenic bacteria could exist in plant from sprouts to growing plant. Noticeable that basil seed was in farm products and it had a chance to be exposed to many sources of contamination such as soil, dirt and unhygienic drying conditions etc.

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While coriander seed was commercial products which expected to be better quality and safety.

Contamination of seeds and seeding can be resulted in the presence of pathogens associated with plants. Immunocytochemical techniques were used to localize internalized *E. coli* O157:H7 expressing green fluorescent protein in germinated mung bean hypocotyl tissue following contamination of intact seeds (Deering *et al*., 2011). The bacteria were found to be associated with every major tissue and corresponding cell type (cortex, phloem, xylem, epidermis, and pith). In addition, the bacteria were localized primarily to the spaces between the cells (apoplast) and not within the cells. Growth experiments performed on mung bean plants found they could support the replication of bacteria to high levels and found 7 log CFU per plant following seed contamination and that these levels could be sustained over a 12-day period. Moreover, Warriner *et al.* (2005) demonstrated that *E. coli* P36 initially introduced on seeds can contaminate the surface of spinach, lettuce, and coriander. Decontaminating seeds, with 50 ppm of ozone or 1000 ppm acidified sodium chlorite and 1000 ppm, and quaternary ammonium salt for 5 min, could reduce the risk of carriage of *E. coli* on the surface of plants. However, proper sanitization would eliminate pathogens from the surface of foods (such as seed) but not inner tissues.

The results of our study showed that soil and irrigation water might be the major source of contamination at pre-harvest environment, particularly water sources from furrow with surface water being used on the sweet basil farm. Water reservoir could be impacted on level of *E. coli* contamination in irrigation water. The population of *E. coli* contamination in irrigation water applied in sweet basil farm showed the higher population than the irrigation water applied in coriander farm since irrigation water used on the coriander farms comes from underground water, which shown less contamination (Table 13).

Islam *et al*, (2004) reported that *E. coli* O157:H7 can be transferred from contaminated soil and water to the surface of lettuce and parsley leaves.

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Oliveira *et al*. (2012) reported that the presence of *E. coli* O157:H7 on outer lettuce leaves was higher than on inner leaves because they are more exposed to environmental conditions and will be contaminated through direct contact with the soil. They observed that there was transfer of pathogen from contaminated soil and irrigation water to edible parts of lettuce leaves. However they did not conclude that the transfer took place by internalization via root system or by direct contact to soil, insects or other vectors.

Escherichia coli can be transmitted from contaminated soil to growing vegetables (Natvig *et al*., 2002). Franz *et al*. (2007) showed the presence of *E. coli* O157:H7 and *S.* Typhimurium in internal or protected subsurface locations of lettuce plants when cultivated in contaminated soil. Then, the contaminated irrigated water used on the farm was suggested as a possible source of the *E. coli* 0157:H7 during pre-harvest (Oliveira *et al*, 2012).

Based on the results from swab samples collected from sweet basil packing houses, the overall sanitation of utensil (gloves, tables, scissors, cover materials and basket) was in an unhygienic condition. All of those were contaminated with *E. coli* (Table 10) and high level of contamination was found in gloves and wash water (20-67% at >100 CFU/g, ml). Basket was contaminated with *E. coli* at 1.47-2.56 log CFU/cm^2 and heavy contamination was found in 60% of them (100-<1000 CFU/cm^2). Although, cover material, 80% (n=4) of samples was not detected (detection limit was 5 cfu/cm^2), but the practices in the packing house indicated a lack of knowledge, particularly of the microbial contamination aspect. Table was contaminated with *E. coli* at 0.48-2.98 log CFU/cm^2 and only 1 sample of scissors was contaminated with *E. coli* (Table 11).

Environmental samples from coriander's packing house showed cover material (n=8) and basket (n=10) were contaminated with *E. coli* with a heavy load ranging from 2.27-4.07 and 2.00-3.77 log CFU/cm^2, respectively (Table 12). Samples of wash water, 90% (n=10) of samples was also heavy contaminated with *E. coli* at 1.06-3.21 log CFU/ml.

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Before, during and after preparation, bare hand and table were taken to enumerate for *E. coli* and found that the contamination ranging from 0.3-3.75 (70%, n=10) and 0.62-3.75 log CFU/cm^2 (60%, n=10).

E. coli contamination on utensil samples used in packing house both basil farm and coriander farm indicated improper cleaning and sanitizing. Besides, the wash water which was taken during washing vegetables was found to have heavy loads of *E. coli* from >10->1000 CFU/ml indicated a poor quality of water which may be used to moisten cover materials and cleaning baskets.

A heavy load of *E. coli* was also found in baskets and cover materials; the heavy contamination which showed indicated the need for appropriate cleaning and sanitizing.

Duffy. (2005) reported *E. coli* was detected on many of utensil surfaces samples (boxing ramps, conveyor belts, plastic bags, and bins used for harvesting, a receiving hopper, trailers used for transport, and an unloading ramp used to move harvested product on the processing line), they found both in the field and in the packing shed of cantaloupe, orange, and parsley. Although the positive samples was low (26 /280, 9.3%), but the overall mean count of *E. coli* on positive samples was quite high as 2.1 log CFU/400 cm^2. *E. coli* was detected most frequently in samples obtained from the surfaces of trailers (7 of 20) unloading ramps (6 of 15) and plastic harvest bags (2 of 5). The equipment used for transport could be contaminated with pathogenic bacteria because they were opened, exposed to dust, road dirt, insects, and rodents and used repeatedly without washing (Geldreich and Bordner, 1971). Some study reported that *E. coli* was not detected on the surface of employee gloves or hands (Parish, 1998; Duffy *et al*., 2005).

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Table 10 Population of *E. coli* (log CFU/g, ml,cm^2) in environmental samples collected from packing house and sweet basil farm

Farm	Period	Source		No. of samples	Positive sample (%)	Range of positive sample	Mean of positive (±SD)
SA	March 2009	Farm	Seed	3	3(100)	4.81-4.86	4.83±0.029
			Soil	15	10 (66.67)	0.70-3.31	1.61±0.74
			Fertilizer	15	1(6.67)	0.70[a]	0.70±0.00 [a]
			Irrigation water	15	9(60)	0.18-2.01	0.77±0.66
		Packing house	Gloves	10	7(70)	0.30-3.04	1.50±0.94
			Table	10	3(30)	0.48-2.98	1.67±1.25
			Scissors	10	1(10)	1.47[a]	1.47±0.00[a]
			Wash water	3	3(100)	1.17-3.07	2.26±0.98
			Cover material	5	1(20)	1.69[a]	1.69±0.00[a]
			Basket	5	5(100)	1.47-2.56	2.07±0.47

[a]: Only one sample positive, can not present range and mean of positive

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Table 11 Percentage (%) of *E. coli* in environmental samples collected from packing house and sweet basil farm

Farm	Period	Samples		No. of samples and percentage (%) of samples in the indicated interval[b]				
				ND[a]	0-<10	10-<100	100-<1000	≥1000
SA	March 2009	Farm	Seed	0	0	0	0	3(100)
			Soil	5(33.33)	1(6.67)	6(40)	3(20)	0
			Fertilizer	14(93.33)	1(6.67)	0	0	0
			Irrigation water	6(40)	5(33.33)	3(20)	1(6.67)	0
		Packing house	Gloves	3(30)	2(20)	3(30)	1(10)	1(10)
			Table	7(70)	1(10)	1(10)	1(10)	0
			Scissors	9(90)	0	0	1(10)	0
			Wash water	0	0	1(33.33)	1(33.33)	1(33.33)
			Cover material	4(80)	0	0	1(20)	0
			Basket	0	0	2(40)	3(60)	0

[a]: Not detected (detection limit was 5 cfu/g,ml,cm^2)

[b]: Range of *E. coli* population (CFU/g,ml/cm^2)

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Table 12 Population of *E. coli* (log CFU/g,ml,cm^2) in environmental samples collected from packing house and coriander farm

Farm	Period	Source		No. of samples	Positive sample (%)	Range of positive sample	Mean of positive (±SD)
CA	July 2009	Farm	Seed	3	0(0)[a]	0.00	0.00[a]
			Soil	15	10 (66.67)	1.17-3.00	2.19±0.77
			Fertilizer	15	0(0)[a]	0.00	0.00[a]
			Irrigation water	15	0(0)[a]	0.00	0.00[a]
		Packing house	Hands	10	7(70)	0.30-3.75	2.12±1.09
			Table	10	6(60)	0.62-3.75	2.83±0.48
			Wash water	10	9(90)	1.06-3.21	2.35±0.31
			Cover material	8	8(100)	2.27-4.07	3.47±0.15
			Basket	10	10(100)	2.00-3.77	2.93±0.27

[a]: Not detected (detection limit was 5 cfu/g

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Table 13 Percentage (%) of *E. coli* in environmental samples collected from packing house and coriander farm

Farm	Period	Samples		No. of samples and percentage (%) of samples in the indicated interval[b]				
				ND[a]	0-<10	10-<100	100-<1000	≥1000
CA	July 2009	Farm	Seed	0	3(100)	0	0	0
			Soil	7(46.67)	0	0	8(53.33)	0
			Fertilizer	15(100)		0	0	0
			Irrigation water	15(100)		0	0	0
		Packing house	Hands	3(30)	1(10)	3(30)	0	3(30)
			Table	4(40)	1(10)	0	1(10)	4(40)
			Wash water	1(10)	0	2(20)	5(50)	2(20)
			Cover material	0	0	0	2(25)	6(75)
			Basket	0	0	0	5(50)	5(50)

[a]: Not detected (detection limit was 5 cfu/g/ml,cm^2)

[b]: Range of *E. coli* population (CFU/g,ml/cm^2)

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Prazak *et al*. (2005) investigated the prevalence of *L. monocytogenes* during production and post-harvest processing in farms and packing sheds in south Texas and found that *L. monocytogenes* was contaminated in 3% (26 of 855) of cabbage, water, and environmental samples. They concluded that contact with packing shed surfaces, such as conveyer belt, may be a source of contamination, highlighting the importance of equipment sanitation.

In this study, many samples indicated that these may be the source of *E. coli* contamination found in this study, such baskets and cover material, trimming table, gloves or bare hand, scissors, basket, and cover material. Those have been traditionally recognized as post-harvest control points for process of pathogens to whole or cut produce (Beuchat and Ryu, 1997). Moreover, when utensil is not effectively cleaned and sanitized, biofilm may develop, providing a reservoir of bacteria with the potential to continuously contaminate produce coming to contact the utensil (Blackman and Frank, 1996; Beuchat, 2002).

However, our experiment was not able to elucidate whether the surface contamination resulted in the transfer of greater microbial loads to the produce during processing or whether the processing surfaces became contaminated due to contact with produce contaminated at the pre-harvest phase. The samples taken in this study were quite small (n = 3 to n = 10 in each taken samples) and only 2 commodities were investigated. Further experiments to follow the agricultural practice on farm to factory of exported produce could be valuable to identify the source of contamination and how to prevent it.

The quality of wash water was very important; in our observation wash water has not been changed during washing process. The microbial load found in wash water in sweet basil process was similar to coriander. The microbial load in wash water for sweet basil was range from 1.17-3.07 log CFU/ml and all samples (n=3) were contaminated with *E. coli*. In case of coriander farm, 9 from 10 samples of wash water were contaminated with *E. coli* with 1.06-3.21 log CFU/g,ml.

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Detection of *E. coli* in the environmental samples at packing house indicates a need for improving cleaning and sanitation protocols to reduce pathogenic bacteria from being transferred to the final product**.**

2.1 Microbiological quality in sweet basil and coriander

The microbiological quality of exported fresh produce throughout the process (March 2009-June 2011) was investigated. The prevalence of *E. coli* contamination in sweet basil from SA, SB and SC farms have a tendency to increase throughout the process (after harvesting through at receiving factory) (Table 14).

The contamination may come from the irrigation system since sweet basil was watered from water in a furrow but coriander was watered by ground water though the springer (overhead) system. Coriander seemed to be cleaner compared to sweet basil, however some markets would have stem attached to root during a sate which may have soil contamination.

Basically, sweet basil and coriander grow in direct contact with soil. When sweet basil is harvested, the branches will be cut at approximately 30 cm above the soil whereas coriander was removed with the roots and entire stems from the soil.

The irrigation water was a furrow system with pond water whereas coriander grows in direct contact with soil and this fresh produce is harvested by harvesting the whole plant including roots from soil. The irrigation system is overhead with ground water.

A total of 249 produce samples was collected throughout the fresh produce process, each type of fresh produce was cultivated, harvested and processed at different times, location, and process, as available for each year. The population of *E. coli* contamination in sweet basil ranged from 1.85-2.19 log CFU/g (Table 14). Twenty to fourty % of sweet basil samples after trimming to after transportation (at receiving factory) from SA, SB and SC farm were over standard (≥100 CFU/g) (Table

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15). After washing, 10-30% of the samples were contaminated with *E. coli* at an unacceptable level (>100 CFU/g). However, after being processed at the factory, only 1 sample was found to be contaminated with *E. coli* with lower level (0.7 log CFU/g).

The prevalence of *E. coli* contamination in coriander from CA, CB and CC farm has a tendency to increase throughout the process (after harvesting to after transportation) (Table 16). The concentration of *E. coli* contamination ranged from 1.16-2.99 log CFU/g. During 2009, 30-90% of samples has *E. coli* population over standard (>100 CFU/g) while in 2011, the quality of samples after trimming from CB and CC farm were better (10-30% over standard).

This phenomenon in coriander can be explained by the farmers having changed their practices by skipping the washing process. They removed the dirt and soil, the root and stem still attached and sent the coriander (without washing) directly to factory. This may have assisted in the reduction of the distribution of *E. coli* and decreased the load found in 2009 to that found in 2011.

However, we could not get rid of *E. coli* completely. *E. coli* was detected more frequently on product samples in the packing house after trimming and washing. Throughout the process the results agreed with previous work, the average *E. coli* levels associated in cantaloupe increased by 2 log CFU/g from beginning to the end of processing (Duffy *et al*., 2005). In many cases, there were no significant changes in the microbiological quality of domestic leafy green and herbs during the packing process, and the initial levels of *E. coli* in these produce items were extremely low. (Johnston *et al.,* 2006).

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Table 14 Population of *E. coli* (log CFU/g) in sweet basil collected from various step throughout the production of different farms

Farm	Period	Sample	No. of samples	Positive sample (%)	Range of positive sample	Mean of positive (±SD)
SA	March 2009	After trimming	10	5 (50)	1.00-2.51	1.92±0.60
		After washing	10	8(80)	1.00-3.10	1.91±0.65
		After transportation (at receiving factory)	10	10(100)	1.70-3.26	2.15±0.64
		Exported fresh produce	15	10(66.67)	0.70-2.20	0.91±0.49
SB	May 2011	After harvesting	10	7(70)	1.00-3.32	2.13±0.90
		After trimming	10	6(60)	1.30-3.63	2.19±0.87
		After washing	10	9(90)	1.30-3.22	2.13±0.62
SC	May 2011	After harvesting	10	8(80)	1.00-3.32	1.85±0.52
		After trimming	10	8(80)	0.70-3.63	2.03±0.81
		After washing	10	9(90)	1.18-3.22	1.93±0.63
SD	June 2011	After transportation (at receiving factory)	21	5(23.81)	0.70-2.00	1.39±0.50
		Exported fresh produce	21	1(4.76)	0.70[a]	0.70±0.00[a]

[a]**:** Only one sample positive, can not present range and mean of positive

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Table 15 Percentage (%) of *E. coli* (log CFU/g) in sweet basil collected from various step throughout the production of difference farm

Farm	Period	Samples	No. of samples and percentage (%) of samples in the indicated interval[b]				
			ND[a]	0-<10	10-<100	100-<1000	≥1000
SA	March 2009	After trimming	5(50)	0	2(20)	3(30)	0
		After washing	2(20)	0	5(50)	2(20)	1(10)
		After transportation (at receiving factory)	0	0	5(50)	3(30)	2(20)
		Exported fresh produce	5(33.34)	2(13.33)	6(40)	2(13.33)	0
SB	May 2011	After harvesting	3(30)	0	3(30)	2(20)	2(20)
		After trimming	4(40)	0	2(20)	2(20)	2(20)
		After washing	1(10)	0	5(50)	3(30)	1(10)
SC	May 2011	After harvesting	2(20)	0	5(50)	3(30)	0
		After trimming	2(20)	0	3(30)	4(40)	1(10)
		After washing	1(10)	0	7(70)	1(10)	1(10)
SD	June 2011	After transportation (at receiving factory)	16(76.19)	2(9.52)	2(9.52)	1(4.76)	0
		Exported fresh produce	20(95.24)	1(4.76)	0	0	0

[a]: Not detected (detection limit was 5 cfu/g)

[b]: Range of *E. coli* population (CFU/g)

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Table 16 Population of *E. coli* (log CFU/g) in coriander collected from various step throughout the production of different farms

Farm	Period	Sample	No. of samples	Positive sample (%)	Range of positive sample	Mean of positive (±SD)
CA	July 2009	After trimming	10	10 (100)	2.17-3.00	2.63±0.23
		After soil removing	5	5(100)	2.47-3.69	2.99±0.49
		After washing	10	10(100)	2.17-3.38	2.87±0.42
		After transportation (at receiving factory)	10	10(100)	2.17-3.95	2.93±0.58
		Exported fresh produce	15	15(100)	0.70-3.13	1.95±0.83
CB	June 2011	After harvesting	10	7(70)	1.70-2.70	2.20±0.71
		After trimming	10	4(40)	0.70-1.70	1.37±0.58
CC	June 2011	After harvesting	10	5(50)	0.70-1.60	1.16±0.34
		After trimming	10	5(50)	1.40-2.74	1.92±0.46
CD	July 2011	After transportation (at receiving factory)	21	13(61.90)	0.70-2.81	1.99±0.74
		Exported fresh produce	21	0(0)	0.00[a]	0.00±0.00[a]

[a]: Not detected (detection limit was 5 cfu/g)

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Table 17 Percentage (%) of *E. coli* in coriander collected from various step throughout the production of difference farm

Farm	Period	Sample	No. of samples and percentage (%) of samples in the indicated interval[b]				
			ND[a]	0-<10	10-<100	100-<1000	≥1000
CA	July 2009	After trimming	0	0	0	9(90)	1(10)
		After soil removing	0	0	0	8(80)	2(20)
		After washing	0	0	0	5(50)	5(50)
		After transportation (at receiving factory)	0	0	0	6(60)	4(40)
		Exported fresh produce	0	3(20)	5(33.33)	5(33.33)	2(13.33)
CB	June 2011	After harvesting	8(80)	0	1(10)	1(10)	0
		After trimming	7(70)	0	1(10)	2(20)	0
CC	June 2011	After harvesting	5(50)	1(10)	4(40)	0	0
		After trimming	4(40)	0	3(30)	3(30)	0
CD	July 2011	After transportation (at receiving factory)	8(38.09)	1(4.76)	5(23.81)	7(33.33)	0
		Exported fresh produce	21(100)	0	0	0	0

[a]: Not detected (detection limit was 5 cfu/g)

[b]: Range of *E. coli* population (CFU/g)

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Total bacteria presented in environmental samples in both pre-harvest and during preparation of vegetables at packing house, although the results varied greatly but the number in overall was quite high particularly in hand, table, wash water and basket (Table 18-19). Seed, soil, and fertilizer from sweet basil farms presented the high amount of total plate count and yeast and mould count, except coriander seed. Coriander seed count was not so high (2.54 log CFU/g) when compared with sweet basil seed (6.2 log CFU/g). We discussed the difference in number may come from the seed production, whether in-farm production or commercial production. We expected that commercial production should be cleaned and treated fungicide, thus cause the microbial count to be lower compared to in-farm seed production.

Irrigation water used the for sweet basil farm presented the high amount of total plate count and yeast and mold count compared to coriander farm due to the different source of irrigation water. Wash water presented the high level of total plate count and in both farms (7.2 to 7.8 log CFU/ml) in vegetables. Normally the wash water should be chlorinated from our survey, none of the farms was applying the sanitizing agent in wash water.

Cover material and baskets used in the packing house were expected to be cleaned and sanitized. The dirty utensil might be a source of contamination, particularly after preparation and vegetable ready to be sent for factory or further process. High number in cover material (7.2-7.7 log CFU/cm^2) and basket (5.1-5.8 log CFU/cm^2) showed the unhygienic condition of cleaning utensil in these farms. High numbers of yeast and mold also represented the improper cleaning and counted as a source of spoilage due to microorganisms.

Overall, the total plate count and yeast and mold count in vegetables were decreased throughout the process (Table 18-19).

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Table 18 Microbiological quality of environmental samples collected from sweet basil, coriander farm and packing house (Farm SA and CA)

Samples	Total plate count (log CFU/g,ml,cm^2)		Yeast and mold (log CFU/ g,ml,cm^2)	
	Sweet basil	Coriander	Sweet basil	Coriander
Seed	6.21±0.25	2.54±0.14	5.18±0.09	2.16±0.78
Soil	7.10±0.35	7.43±0.34	4.76±0.39	4.95±0.37
Fertilizer	8.24±0.63	7.47±0.33	6.46±1.02	3.46±0.57
Irrigation water	4.16±0.49	1.67±1.20	2.06±1.08	0.10±0.16
Gloves/Bear hands	6.84±1.16	7.96±0.81	3.93±0.97	3.49±0.65
Table	5.45±1.59	7.19±1.10	2.25±0.76	3.06±0.81
Wash water	7.13±0.26	7.81±1.57	4.97±0.09	3.92±0.95
Cover material	5.01±0.16	5.79±0.50	2.84±0.51	1.91±0.99
Basket	7.19±0.12	7.63±1.16	3.36±0.41	3.34±0.53

Table 19 Microbiological quality of sweet basil and coriander collected from various step throughout the production of difference farm (Farm SA and CA)

Samples	Total plate count (log CFU/g)		Yeast and mold (log CFU/g)	
	Sweet basil	Coriander	Sweet basil	Coriander
After trimming	5.96±0.53	8.49±0.48	5.54±0.59	5.16±0.20
After soil removing	NA[a]	8.35±0.68	NA	5.22±0.34
After washing	5.43±0.70	7.47±0.65	5.39±0.45	4.79±0.43
After transportation (at receiving factory)	6.14±0.36	7.31±0.44	5.38±0.42	4.73±0.37
Exported fresh produce	5.45±1.59	6.25±0.27	4.27±0.43	4.40±0.19

[a]: Not analyzed

The total plate count on sweet basil and coriander samples throughout the process were decreased from 6.3 to 5.4 and 8.5 to 6.2 log CFU/g, respectively. Yeast and mold of these samples throughout the process were also decreased but vegetables still have a high number in exported product. The total aerobic count and yeast and mold of sweet basil and coriander samples after washing tended to be decreased. The

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cross contamination may occur during trimming, washing step, and further process. Additionally, Parish and Higgines (1990) reported that total microbial counts increased on commercially prepared grapefruit sections during processing indicating a buildup of contamination on the processing utensil.

2.2 Prevalence of pathogenic *E. coli* in sweet basil and coriander

One hundred and eighty four samples of produce collected in 2011 (sweet basil, n=102; coriander, n=82) were analyzed for the presence of *E. coli* O157:H7. Of the 99 isolates (48 isolates from sweet basil, 51 isolates from coriander) were identified. None of them were *E. coli* O157:H7 (Table 20).

Table 20 Number of *E. coli* isolates from sweet basil and coriander collected from 6 farms (SB, SC, SD, CB, CC and CD)

Sample	No. of isolated samples (% of positive) collected from 6 farms	
Sweet basil (n[a]=48)	SB(n=23)	SC(n=25)
After harvesting	6(0)	7(0)
After trimming	4(0)	6(0)
After washing	5(0)	7(0)
After transportation (at receiving factory)	3(0)	3(0)
Export	5(0)	2(0)
Coriander (n=51)	CB(n=27)	CC(n=24)
After harvesting	10(0)	9(0)
After trimming	10(0)	7(0)
After transportation (at receiving factory)	4(0)	4(0)
Export	3(0)	4(0)

[a]: Number of isolates

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Similarly results in March, 2000, the USFDA conducted another produce survey including 1028 domestic samples of high volume produce such as cantaloupe, celery, cilantro, loose-leaf lettuce, parsley, scallions, strawberries and tomatoes. 99% of produces were found to be free of *Shigella* and *Salmonella* spp. Moreover, none of these produce items were contaminated with *E. coli* O157:H7. Of the 11 contaminated samples, 6 (55%) were contaminated with *Salmonella* spp. and 5 (45%) were contaminated with *Shigella* (USFDA, 2003).

Moreover, 890 samples of fresh produce(such as lettuce, pre-cut salad) and 130 samples of domestic growing herbs (basil, chervil, coriander, dill, lemon, etc) in Norway were tested, none of domestic growing herb samples were found positive for *Salmonella* spp. and *E. coli* O157 (Johannessen *et al.,* 2002).

It has been reported as *E. coli* O157:H7 contaminated in coriander and cilantro (Beuchat 1996). Later, in March 1999, the U.S. Food and Drug Administration (USFDA) conducted a survey of 1003 samples which imported from 21countries including broccoli, cantaloupe, celery, cilantro, loose-leaf lettuce, parsley, scallions, strawberries and tomatoes. Samples were analyzed for *Salmonella* spp., *Shigella* and *E. coli* O157:H7. Four % (44 of 1003 samples) were contaminated with either *Salmonella* or *Shigella.* The produce with the most contamination of *Salmonella* spp. and *Shigella* was cantaloupe and cilantro (USFDA, 2001). Exported fresh produce was made to indicate that they contained the pathogenic bacteria and may cause illnesses to consumer.

Freshly cut salad greens largely contained members of the bacterial genera found in water and soil (Shapiro and Holder, 1960). From our observation, there are many possible sources of contamination on fresh produce in the packing house not only the contamination from the pre-harvest stage. This fresh produce is exposed to many hygienic utensil and conditions during trimming, washing, packing, and extensive handling. The implementation of good hygienic practices needs to be done with strict control, proper training, and the understanding of the microorganisms in foods.

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3 To evaluate the cross contamination between washing process and the effect of chlorine on reduction of *E. aerogenes* contamination

In this experiment, 50 ppm of available chlorine was applied in wash water to determine the efficiency of chlorine to reduce *E. aerogenes* in water, using in washing process. The experiment was also to determine whether shaking will influence removing bacteria from produce, by comparing gentle shaking by hand and without shaking. Washing basil in chlorinated water with and without shaking for 5 min resulted in 1.12 and 1.19 log unit reduction from the initial load of ~4 log CFU/g (Table 21), extend the period of washing of 10 and 15 min showed no significant difference (α=0.05) in microbial reduction. *E. aerogenes* count in all chlorinated water was not detected at washing time (5, 10, and 15 min). Chlorinated water assisted to inhibit the cross contamination between inoculated vegetables and wash water, and expected to prevent cross contamination to further process.

Similar results were shown in coriander, the level of *E. aerogenes* contamination decreased when 50 ppm chlorine was applied to the wash water, resulting in 1.29 and 1.35 log unit reduction in sample with and without shaking at 5 min. At 5 min is likely to be proper washing time to decontamination by applying chlorine 50 ppm. If the load of pathogenic bacteria in Enterobacteriaceae such as *E. coli*, is not higher than 100 CFU/g, since the highest reduction in this study indicated at 1.35 log or 32 colony. Thus, the level of contamination in fresh produce is very crucial in order to enhance the safety of this commodity. Previous results showed the load of *E. coli* in basil and coriander is commonly high number after harvesting, the strictly best practices in packing house should be done in order to reduce the bacteria load in fresh produce before entering food plant. A few hundred of *E. coli* contamination at receiving factory assists to manage the load reduction in processing at factory. Therefore fresh produce for export will be able to manage to have less count than 100 CFU/g as amended in the regulation.

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Table 21 *E. aerogenes* count* on vegetable and wash water after washed with reused water *E. aerogenes* count on inoculated vegetables and wash water with and without shaking

Condition	Sodium hypochlorite	Samples	Washing time			
			Initial load	5 min	10 min	15 min
Shaking	No	Basil	4.17±0.14^{Aa}	3.51±0.20^{Aa}	3.63±0.10^{Aa}	3.53±0.25^{Aa}
		Wash water	ND^{Aa} **	2.97±0.22^{Aa}	3.06±0.15^{Aa}	3.08±0.23^{Aa}
	50 ppm	Basil	4.17±0.14^{Aa}	2.98±0.09^{Ba}	2.92±0.08^{Ba}	2.88±0.08^{Ba}
		Wash water	ND^{Aa}	ND^{Ba}	ND^{Ba}	ND^{Ba}
Without shaking	No	Basil	4.17±0.14^{Aa}	3.64±0.14^{Aa}	3.64±0.09^{Aa}	3.65±0.13^{Aa}
		Wash water	ND^{Aa}	3.11±0.31^{Aa}	2.95±0.54^{Aa}	3.17±0.36^{Aa}
	50 ppm	Basil	4.17±0.14^{Aa}	2.98±0.11^{Ba}	2.89±0.13^{Ba}	2.82±0.16^{Ba}
		Wash water	ND^{Aa}	ND^{Ba}	ND^{Ba}	ND^{Ba}
Shaking	No	Coriander	4.21±0.22^{Aa}	3.51±0.14^{Aa}	3.70±0.26^{Aa}	3.49±0.16^{Aa}
		Wash water	NA^{Aa}	2.85±0.35^{Aa}	2.92±0.16^{Aa}	2.98±0.25^{Aa}
	50 ppm	Coriander	4.21±0.22^{Aa}	2.92±0.16^{Ba}	2.82±0.20^{Ba}	2.68±0.35^{Ba}
		Wash water	ND^{Aa}	ND^{Ba}	ND^{Ba}	ND^{Ba}
Without shaking	No	Coriander	4.21±0.22^{Aa}	3.52±0.15^{Aa}	3.55±0.08^{Aa}	3.55±0.21^{Aa}
		Wash water	ND^{Aa}	2.73±0.45^{Aa}	2.59±0.21^{Aa}	2.54±0.14^{Aa}
	50 ppm	Coriander	4.21±0.22^{Aa}	2.86±0.21^{Ba}	2.84±0.24^{Ba}	2.74±0.31^{Ba}
		Wash water	ND^{Aa}	ND^{Ba}	ND^{Ba}	ND^{Ba}

*: All values are mean (log CFU/g,ml) ± SD of data from 3 replicates independent experiments with 2 samples analyzed per replicate (n=6)
**: Not detected (limit of detection was 1 CFU/ml wash water)
A-B: Comparison of the effect of sanitizing agent, within the same vegetable type and washing time, values in the same column that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$)
a-b: Comparison of the effect of shaking force, within the same vegetable type and washing time, values in the same column that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$)

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According to the results between reduction with and without shaking, this study showed no significant differencere of *E. aerogenes* in these vegetables and no significant different in cross contamination from vegetables to water, particularly, samples without chlorine. In this study, the shaking force by hand might not be enough to achieve to the release of the attachment of *E. aerogenes* from vegetables surface. The population of *E. aerogenes* after washing with and without shaking on vegetables (washing without chlorine) were not significant difference (α=0.05).

On the contrary, Parnell and coworker demonstrated that washing melons by soaking and scrubbing with brush scrub can reduce *Salmonella typhimurium* on melons more than soaking alone by 0.9 log CFU, since the hardly scrub method can be applied at the outer surface of melon (Parnell *et al*., 2005). However limitation was noted that this scrubbing might be helpful in hard surface but not in soft texture like leafy vegetable.

Failure of shaking method to remove *E. aerogenes* may explain as bacteria could hide and survive in protective hydrophobic pockets or folds in the leaf surface (Adams *et al*., 1989), particularly basil and coriander, which are leafy vegetables with many folds. The characteristic of basil and coriander leaves show the soft texture, thus the scrub technique mentioned as above is not the suitable method to apply.

Seymour *et al.,* (2002) showed the different washing technique to reduce the population of *S.* Typhimurium attached to iceberg lettuce. Washing with water, chlorinated water (25 ppm) and ultrasound were 0.7, 1.7, and 1.5 log reduction. The addition of chlorine to water (25 ppm) combined with power ultrasound could reduce *S.* Typhimurium by 2.7 log CFU. Attached or entrapped *S.* Typhimurium are not readily accessible to chlorine. When ultrasound was applied, the capitation enhances the mechanical removal of attached or entrapped bacteria on the surfaces of vegetable by displacing or loosening particles through a shearing or scrubbing action, then *S.* typhimurium is exposed to wash water.

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The levels of *E. aerogenes* on sound basil and sound coriander after washing in reused water for 5 min (Table 22), they were reaching to 2.23 and 2.31 log CFU/g, respectively (2nd time). After washing each 5 min, the third and fourth washing processes were done with sound vegetables, *E. aerogenes* counts in reused water (without chlorine) were 3 log CFU/ml, with no significant difference (α=0.05) between 2nd (2.23 log CFU/g), 3rd (2.27 log CFU/g) and 4th (2.58 log CFU/g) washing. In case of coriander, the *E. aerogenes* counts in the 2nd, 3rd and 4th of wash water were similar count as ~3 log CFU/g with no significant difference (α=0.05). When adding 50 ppm sodium hypochlorite in wash water, noticeably resulted in reduction of *E. aerogenes* in inoculated basil and coriander by 1.25 (from initial load 4.17 log CFU/g) and 1.03 log CFU/g (from initial load 4.21 log CFU/g), respectively. None of vegetables and wash water which applying chlorine (5 min) was detected *E. aerogenes*, the available chlorine was tested and found remaining in wash water (as free chlorine 32.6 ppm at 5 min).

The results indicated that washing vegetables with 50 ppm sodium hypochlorite could prevent the cross contamination between wash water even though the process uses the same batch of washing water for 4 times within 20 min.

Regardless of the previous results, the level of chlorine in chlorinated water (50 ppm) was not enough to eliminate *E. aerogenes* attached to vegetables, but the efficacy of chlorine showed the potential to kill planktonic cells (floating) in water after removing from the contaminated vegetables. Adding chlorine as the sanitizer was needed in washing process for killing pathogenic bacteria and preventing the cross contamination between contaminated vegetables through the wash water to sound vegetables.

To evaluate the potential of cross-contamination during washing process while inoculated basil and sound basil were simultaneously washed in the same batch, the experiment was conducted to determine whether chlorine will assist to prevent the contamination among these vegetables.

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Initial *E. aerogenes* levels of inoculated basil and coriander before washing were 4.17 ± 0.14 and 4.21 ± 0.22 log CFU/g, respectively (Table 23). After washing in tap water without chlorine for 5 min, *E. aerogenes* count in inoculated basil was 3.75 log CFU/g reduced from the initial load by 0.42 log CFU/g. The level of *E. aerogenes* count in sound basil and wash water were increased to 2.17 and 2.78 log CFU/g, respectively.

When extending period of washing from 5, 10 to 15 min, the level of *E. aerogenes* on sound basil (which now contaminated) and wash water slightly increased with no significant difference. Similar results were observed in coriander washing, this information may indicate that cross contamination between wash water and vegetables occurred while unclean (contaminated) and clean vegetable were washed simultaneously.

Referring to the results from Table 23 showed that the cross-contamination between basil and wash water occurred but it was time independent. Previous research found that *E. coli* cells were able to attach to the surface of the lettuce after 1 min when dipping in the pre-washing tank containing high level of organic matter, and after that cells were already protected depending on the nature of plant from the physical removal by water (López-Gálvez *et al*., 2010).

When 50 ppm of available chlorine was applied, washing basil and coriander in chlorinated water without shaking for 5 min, resulted in 1.19 and 1.26 log unit reduction, respectively. Extending the period of washing to 15 min, the reduction of *E. aerogenes* in inoculated basil and inoculated coriander were not increased with no significant difference when compared to the earlier washing (α=0.05). Chlorine has obviously affected on *E. aerogenes* reduction and prevents the contamination of either vegetables or water. All sound basil and coriander, including chlorinated water, were not detected to be contaminated with *E. aerogenes* throughout simultaneously washing for 15 min.

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Table 22 Transfer of *E. aerogenes** on vegetables and wash water after washed with reused water

Sequence of washing	Treatment							
	0 ppm NaOCl (5 min)		50 ppm NaOCl (5 min)		0 ppm NaOCl (5 min)		50 ppm NaOCl (5 min)	
	Basil	Wash water	Basil	Wash water	Coriander	Wash water	Coriander	Wash water
Initial load	4.17±0.14 ^{Aa}	ND ^{Ab} **	4.17±0.14 ^{Aa}	ND ^{Aa}	4.21±0.22 ^{Aa}	ND ^{Ab}	4.21±0.22 ^{Aa}	ND ^{Aa}
1st washing	3.54±0.28 ^{Ab}	2.97±0.10 ^{Aa}	2.92±0.12 ^{Bb}	ND ^{Ba}	3.70±0.52 ^{Ab}	3.05±0.05 ^{Aa}	3.18±0.07 ^{Bb}	ND ^{Ba}
2nd washing	2.23±0.32 ^{Ac}	3.01±0.16 ^{Aa}	ND ^{Bc} ***	ND ^{Ba}	2.31±0.28 ^{Ac}	3.05±0.01 ^{Aa}	ND ^{Bc}	ND ^{Ba}
3rd washing	2.27±0.35 ^{Ac}	3.01±0.13 ^{Aa}	ND ^{Bc}	ND ^{Ba}	2.25±0.13 ^{Ac}	3.03±0.04 ^{Aa}	ND ^{Bc}	ND ^{Ba}
4th washing	2.58±0.25 ^{Ac}	3.02±0.22 ^{Aa}	ND ^{Bc}	ND ^{Ba}	2.60±0.18 ^{Ac}	3.05±0.14 ^{Aa}	ND ^{Bc}	ND ^{Ba}

*: All values are mean (log CFU/g,ml) ± SD of data from 3 replicates independent experiments with 2 samples analyzed per replicate (n=6)

**: Not detected (limit of detection was 1 CFU/ml wash water)

***: Not detected (limit of detection was 5 CFU/g vegetable)

$^{A-B}$: Comparison of the effect of sanitizing agent, with in the same sequence of washing time and vegetable type, values in the same row that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$)

$^{a-c}$: Comparison of the effect of washing sequence, with in the same treatment and vegetable type, values in the same column that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$)

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Table 23 Transfer of *E. aerogenes** on inoculated vegetables, sound vegetables and wash water after simultaneously washed together for 5, 10 and 15 min

Sodium hypochlorite	Samples	Washing time			
		Initial load	5 min	10 min	15 min
No	Contaminated basil	4.17 ± 0.14^{Aa}	3.75 ± 0.09^{Ab}	3.70 ± 0.14^{Ab}	3.66 ± 0.08^{Ab}
	Sound basil	ND^{Bb}	2.17 ± 0.26^{Aa}	2.25 ± 0.31^{Aa}	2.34 ± 0.18^{Aa}
	Wash water	ND^{Bb}	2.78 ± 0.23^{Aa}	2.71 ± 0.11^{Aa}	2.69 ± 0.11^{Aa}
50 ppm added	Contaminated basil	4.17 ± 0.14^{Aa}	3.05 ± 0.06^{Bb}	2.95 ± 0.07^{Bb}	2.91 ± 0.08^{Ab}
	Sound basil	ND^{Ba}	ND^{Ba}	ND^{Ba}	ND^{Ba}
	Wash water	ND^{Ba}	ND^{Ba}	ND^{Ba}	ND^{Ba}
No	Contaminated coriander	4.21 ± 0.22^{Aa}	3.60 ± 0.19^{Ab}	3.61 ± 0.31^{Ab}	3.64 ± 0.28^{Ab}
	Sound coriander	ND^{Bb}	1.96 ± 0.45^{Aa}	2.10 ± 0.46^{Ba}	2.18 ± 0.31^{Aa}
	Wash water	ND^{Bb}	2.71 ± 0.37^{Aa}	2.93 ± 0.40^{Ba}	2.93 ± 0.40^{Aa}
50 ppm added	Contaminated coriander	4.21 ± 0.22^{Aa}	3.02 ± 0.25^{Bb}	2.95 ± 0.19^{Ab}	2.75 ± 0.47^{Bb}
	Sound coriander	ND^{Ba}	ND^{Ba}	ND^{Ba}	ND^{Ba}
	Wash water	ND^{Ba}	ND^{Ba}	ND^{Ba}	ND^{Ba}

ND: Not detected (limit of detection was 1 CFU/ml, wash water 5 CFU/g vegetable)

*: All values were mean (log CFU/g,ml) ± SD of data from 3 replicates independent experiments (2 samples analyzed per replicate, n=6)

A-B: Comparison of the effect of sanitizing agent, with in the same sequence of washing time and vegetable type, values in the same column that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$)

a-b: Comparison of the effect of washing time, with in the same sanitizing agent and vegetables type, values in the same row that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$)

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4. Probabilistic distribution of *E. coli* exposure on sweet basil and coriander

To simplify the probabilistic model, *E. coli* was assumed as randomly distributed throughout the process (from farm through to receiving factory). The concentration of *E. coli* in fresh produce at the receiving factory was calculated from the log % transfer rate between contaminated and sound vegetables via wash water during the washing process. Calculation was conducted from Equation 1 derived. Then the percentage of transfer rate was transformed into logarithm form. Finally, log % transfer rate distribution was calculated by using @Risk™ software package version 4.5.1 (Palisade, Inc, 2003). Result is shown in Table 24.

Table 24 Transfer rate distribution

Log % transfer rate	Distribution
Tv_cw*	RiskNormal(1.35, 0.23)
Twv_s**	RiskNormal(0.80, 0.16)

*: Tv_cw is the log % transfer rate of *E. aerogenes* from contaminated vegetable to wash water

*: Twv_s is the log % transfer rate of *E. aerogenes* from wash water to sound vegetable

The probabilistic distribution of microbial contamination in sweet basil and coriander was simulated by using @Risk™ program. To investigate which risk factors will influence the population of *E. coli* contamination on fresh produce at the receiving factory, the simple approach model was developed. The overview of simulation variables and parameters were summarized in the Excel spreadsheet used for predicting the population of *E. coli* contamination in vegetables (Table 25). The first column represented the spreadsheet cell designation of the variable on that line of the table. The second column is a text description of the variable. The third and forth columns represented the values of sweet basil and coriander in terms of distribution, respectively. The output of this model was expected to be the population of *E. coli*

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contamination in vegetables after transportation or at the receiving factory (as shown in log CFU/g unit).

4.1 Assumption of the simple approach model

Trimming process

Previous results demonstrated that *E. coli* could be detected in utensils (scissors), hands/gloves and tables used in the packing house. Cross contamination during the trimming process occurred leading to increase not only the population of *E. coli* in vegetables but also the contaminated portions. We assumed that *E. coli* could grow during preparation or pre-processing (Cell B2 in Table 25) at the packing house and during transportation (Cell B11 in Table 25). The population of *E. coli* contamination in vegetables after the trimming step was assumed to be increase, therefore uniform distribution (minimum, maximum) was performed. Weight of vegetables before and after trimming was not considered in this model.

Washing process

The washing process in the packing house assists to remove the soil and dust. Moreover, this step should reduce the microbial load in the vegetable. However, from our observation, the wash water either in sweet basil or coriander have not changed during washing process in 2 hours, therefore reused water could cause the cross contamination. We assumed that the population and portion of *E. coli* contamination in vegetables after washing was increased. The increasing contaminated portion in washing process was assumed as the difference between prevalence of *E. coli* found in vegetables before and after washing (Cell B7 in Table 25). During washing process, the transfer rate was calculated by using data from the washing experiment (Cell B8 in Table 25). The data of inoculated vegetables and sound vegetables, reused water in washed in the same batch, were used to calculate the log % transfer rate (See Appendices A).

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Transportation

From our results, the basket and cover material showed heavy load of *E. coli*. We assumed that contamination could occur during transportation. Difference of *E. coli* contamination in vegetable before and after transportation during handling was assumed as uniform distribution (minimum, maximum).

Danyluk and Schaffner (2011) developed the growth rate of *E. coli* O157:H7 during retail and home storage by using the literature data for *E. coli* O157:H7 growth on leafy green with the temperature range from above $0^{o}C$ to $30^{o}C$ and the results showed that the temperature will support the growth of *E. coli* O157:H7, the population of *E. coli* O157:H7 may increase by as much 1 log CFU/day. Furthermore, the transportation time was observed in this study, only 2 hours at ambient temperature was found. It might support the pathogenic bacteria during transportation with small amounts of pathogenic bacteria. Then the difference of the population of *E. coli* at the receiving factory and after washing (before transportation) was calculated. The probabilistic distribution was assumed as the uniform distribution with minimum and maximum value of the difference population of *E. coli* at the receiving factory and after washing.

4.2 Model simulation

The model was stimulated by using @Risk^{TM} program with 10000 iterations. The results from the simple approach model and regression model were conducted to investigate the risk factors that influence the population of *E. coli* on fresh produce at receiving. The population and prevalence distribution of *E. coli* were fitted by using @Risk^{TM} program. The variation and uncertainty of the input parameters are included in the probability distribution. The suitable probabilistic distribution was chosen when the distribution was lower chi-square value and P-value.

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Simple approach model

The data used in the simple approach model was provided from this survey and washing experiment. The population and prevalence of *E. coli* in vegetables after harvesting, after trimming, and after washing were provided from the microbiological survey in 2011. Because the data obtained in 2011 represents the updated situation. The log % transfer rate was calculated from the washing experiment data. The important variables were the population of *E. coli* contamination in sweet basil after washing, the prevalence of *E. coli* contamination in sweet basil before and after washing, the bacterial transfer rate from contaminated vegetables to wash water, and wash water to sound vegetables.

The population of *E. coli* contamination in sweet basil after washing (Cell B9 in Table 25) was combined between the population of *E. coli* contamination in sweet basil after trimming and increased contamination portion of *E. coli* in sweet basil during washing step. The increasing portion was calculated from the difference in prevalence of *E. coli* contamination in sweet basil before and after washing at packing house (RiskBeta distribution, Cell B7 in Table 25) multiplied by the population of *E. coli* contamination in sweet basil after trimming (before washing, Cell b3 in Table 25) and log % transfer rate of bacteria from contaminated vegetable to wash water and wash water to sound vegetables during washing step (Cell B8 in Table 25).

The simple approach model was conducted to estimate the population of *E. coli* at the receiving factory and investigate the factors that influence on the population of *E. coli* in sweet basil and coriander in this study. The result of sensitivity analysis was carried out using @RISK^{TM} software, Tornado chart presented as horizontal bars. These bars were plotted for each input of variable, with the length of the bars representing the degree of correlation with the mean log CFU of *E. coli* contamination in vegetables (once received at factory) (Figure 19). The longer bar showed the greater sensitivity of variables to the population of *E. coli* in vegetables. The input factors, located in the left hand side of chart, showed the

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positive influence on reducing the population of *E. coli* in vegetables, ordered from largest to smallest (on the bottom). The bars (input factor) located on the right hand side of chart present the negative influence on supporting the growth or increasing of population of *E. coli* contamination in vegetables (output factor).

The regression and correlation sensitivity analysis of input parameter model of *E. coli* contamination in sweet basil at the receiving factory was ranked as the population of *E. coli* contamination in sweet basil after harvesting (*E. coli* load on vegetables in pre-harvest) (0.828). The difference of *E. coli* population in sweet basil during transportation was the second factor's influence on the *E. coli* population in sweet basil particularly when received at the factory. This graph may explain that the prevalence of *E. coli* in sweet basil after the trimming step (0.338) was also concerned as one of the high influence on *E. coli* contamination on basil. The negative factors were listed as the prevalence of *E. coli* in sweet basil before washing, log% transfer rate of bacteria during washing step.

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Table 25 Overview of simulation variables and parameters

Cell	Variable	Value		Unit
		Sweet basil	Coriander	
	Trimming step			
B1	Population of *E. coli* after harvesting	RiskNormal(1.49, 1.07)	RiskBetaGeneral(0.12, 0.39, 0, 2.7)	log CFU/g
B2	Difference during trimming	RiskUniform(-1,0.51)	RiskUniform(0,1.4)	log CFU/g
B3	Population of *E. coli* after trimming	B1+B2	B1+B2	log CFU/g
B4	Washing step			
B5	Prevalence of *E. coli* contamination before washing	RiskBeta(15, 7)	-	-
B6	Prevalence of *E. coli* contamination after washing	RiskBeta(19, 3)	-	-
B7	Difference between prevalence of *E. coli* before and after washing	B6-B5	-	-
B8	Log % Transfer rate[a]	(Tws×Tcw)	-	-
B9	Population of *E. coli* after washing	B3+[B3× (B6×B7)]	-	log CFU/g
B10	Transportation step			
B11	Difference during handling	RiskUniform(-2.18,0)	RiskUniform(0,1.3)	log CFU/g
B12	Population of *E. coli* after transportation (at receiving factory)	B9+B11	B3+B11	log CFU/g

[a]: Tv_cw is the log % transfer rate of *E. aerogenes* from contaminated vegetable to wash water

: Twv_s is the log % transfer rate of *E. aerogenes* from wash water to sound vegetable

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In case of coriander, it was agreed as the population of *E. coli* contamination in coriander after harvesting (0.86) influence on *E. coli* population in sweet basil particularly at receiving factory. The difference of *E. coli* population during trimming (0.374) was the second and the difference of *E. coli* population during transportation has less influence compared to the previous factors.

From simple approach model, the predicted value of *E. coli* population contaminated in sweet basil and coriander was 0.40 and 1.98 log CFU/g, respectively shown in Figure 20 with the observed population of *E. coli* contaminated in sweet basil and coriander at receiving factory at 0.35 and 1.23 log CFU/g, respectively. The difference of *E. coli* population in sweet basil between the predicted and observed values might be caused from the variation of samples such as the small sample numbers were collected from each step at packing house, only 20 samples were collected in each step and used to predicted the population of *E. coli* contamination at receiving factory. The transfer rate was calculated which is based on extrapolation with the fixed *E. aerogenes* contaminated load. The initial load of vegetable samples taken from packing house might vary. In addition, the transfer rate was calculated from simply equation, only concentration of *E. aerogenes* was calculated, other factors were not included such as time. Moreover, the taken samples might be contaminated before washing since it might lead to increase the contamination load in wash water. Those 2 data sets (population of *E. coli* contaminated after washing and at receiving factory) have not relevance due to they were collected from difference farm and difference period of time. The model requires validation method. Further refinements in the estimates of these variables (such as population of *E. coli* contamination in vegetables in each step) as well as any of the data derived from the published literature would help to improve our ability to estimate the population of *E. coli* contamination in the sweet basil at receiving factory.

The results from tornado curve (Figure 19) indicated that microbiological quality of vegetables before entering each step of process is crucial to the quality and safety of product. For example, the prevalence of *E. coli* on vegetables after harvesting, after trimming, after washing, and after transportation will influence on

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the load of *E. coli* on vegetables entering food processing plant. However, the prevalence of *E. coli* contamination before washing seem to show the negative impact on the load of *E. coli* on vegetables before entering food processing, which may explain that the distribution of *E. coli* contamination of vegetables carry over from farm through trimming process will be less compared to the distribution of *E. coli* during washing process. Water assists as the media to distribute cells, therefore the chance of positive prevalence will be higher in washed vegetables compared to unwashed vegetables. To inhibit the cross contamination during washing, the sanitizing agent should be added in wash water.

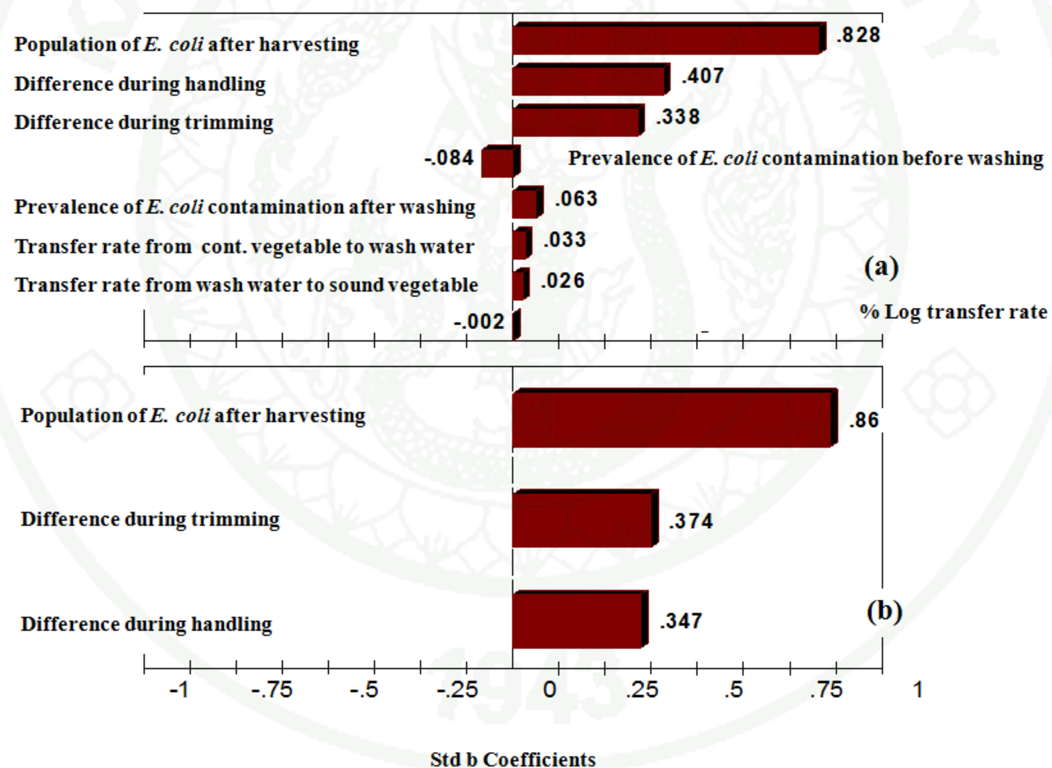

Figure 19 Sensitivity analysis of *E. coli* contamination in sweet basil (a) and coriander (b) after harvesting to transportation to factory

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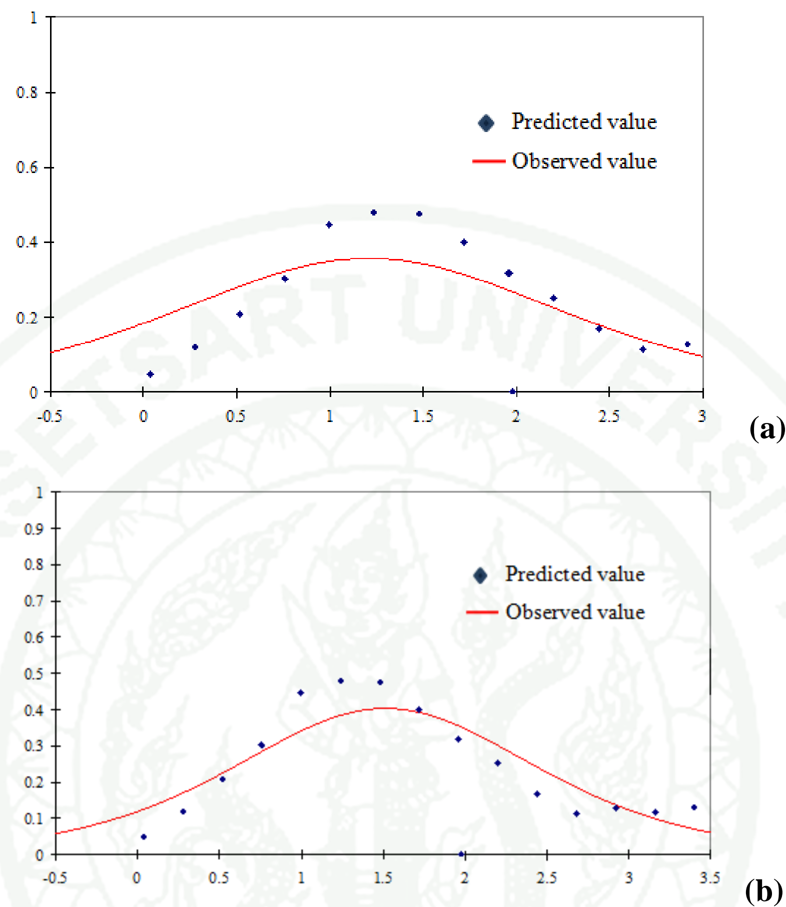

Figure 20 Observed and predicted population of *E. coli* in sweet basil (a) and coriander (b) when using simple approach model

Regression model

The other approach to investigate whether factors influence on the population of *E. coli* contaminated on vegetable at the receiving factory was the regression model. Regression model was built in Excel spread sheet by using the population of *E. coli* contamination in vegetables from our research. We assumed the distribution of *E. coli* load in vegetables found in samples during preparation in the packing house was directly correlated to the load of *E. coli* on vegetables at receiving factory. The regression model of population of *E. coli* contamination in sweet basil at receiving factory was 0.935+(0.134×population of *E. coli* contamination in sweet basil after harvesting)-(0.058×population of *E. coli* contamination in sweet basil after

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trimming)+(0.619×population of *E. coli* contamination in sweet basil after washing) and of coriander was 0.454-(0.251×population of *E. coli* contamination in coriander after harvesting+(1.267×population of *E. coli* contamination in coriander after trimming), respectively (Table 26).

Table 26 Variable and value of regression model

Variable	Values		Unit
	Sweet basil	Coriander	
After harvesting	RiskNormal(1.49, 1.07)	RiskBetaGeneral(0.12, 0.39, 0, 2.7)	log CFU/g
After trimming	RiskLogistic(1.61, 0.76)	RiskBetaGeneral(0.12, 0.39, 0, 2.7)	log CFU/g
After washing	RiskLogistic(1.85, 0.46)	-	log CFU/g
Model[a]	Receiving = 0.935+(0.134×After harvesting)-(0.058×After trimming)+(0.619×After washing)	Receiving = 0.454-(0.251×After harvesting+(1.267×After trimming)	log CFU/g

[a]: Receiving = Population of *E. coli* contamination in sweet basil or coriander at receiving factory

: After harvesting = Population of *E. coli* contamination in sweet basil or coriander after harvesting

: After trimming = Population of *E. coli* contamination in sweet basil or coriander after trimming

: After washing = Population of *E. coli* contamination in sweet basil after washing

The sensitivity analysis of input parameter model of population of *E. coli* contamination in sweet basil at the receiving factory (Figure 21) was ranked as the population of *E. coli* contamination in sweet basil after washing (0.953), as well as the population of *E. coli* contamination in sweet basil after harvesting (0.258), the population of *E. coli* contamination in sweet basil after trimming (-0.133) was less of an influence on *E. coli* load in sweet basil compared to the previous factors. In case of coriander, surprisingly the population of *E. coli* contamination in coriander after

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trimming (0.984) was ranged as the greatest influence on *E. coli* load on vegetable at the receiving factory. The population of *E. coli* contamination in coriander after harvesting (-0.175) was considered to have less impact.

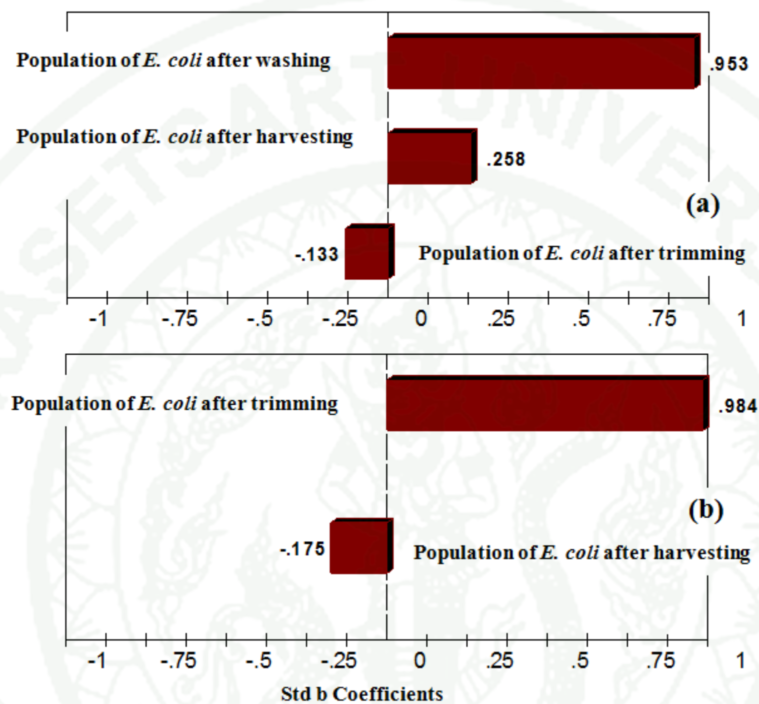

Figure 21 Sensitivity analysis of *E. coli* in sweet basil (a) and coriander (b) at receiving before entering factor performed by regression analysis

The predicted value of *E. coli* population in contaminated sweet basil and coriander was 0.30 and 1.17 log CFU/g, respectively with the observed population of *E. coli* in contaminated sweet basil and coriander at 0.35 and 1.23 log CFU/g, respectively. The difference between the predicted and observed value was small as shown in Figure 22. Then, the simple approach model might be used to predict the population of *E. coli* in contaminated vegetables. However the model should be validated in further research.

The population of *E. coli* contamination in coriander after trimming influenced the population of *E. coli* contamination in coriander at the receiving factory with

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bigger impact when compared with the population of *E. coli* contamination in coriander after harvesting. During trimming process, the spreading of *E. coli* from soil to vegetables was occurred. The soil-contracted root was removed by hands' workers. After that the hands' workers carry the coriander.

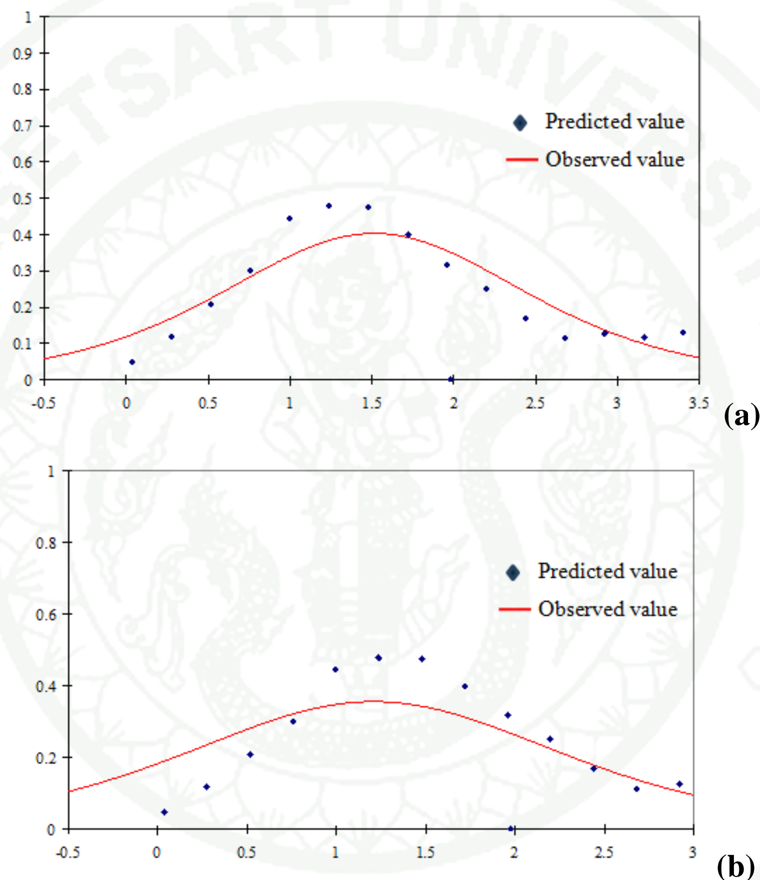

Figure 22 Observed and predicted population of *E. coli* in sweet basil (a) and coriander (b) when using regression model

Using regression models could identify the population of *E. coli* in vegetables at the receiving factory which is the raw material for process at factor, is the most important quality to be considered. It was supported that the most factor affecting the population of *E. coli* contamination in vegetables upon the factory's receiving vegetables was the population of *E. coli* contamination at farm production (or pre-harvest). Managing the initial load in the field or production farm is difficult

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to conduct because there a lot of factors involved, but it needs to be done. Previous data showed that the quality of soil and irrigation water plays an important role in food safety aspects in the field as well as implementation of GAPs. The addition approach to reduce the population of *E. coli* contamination in finished produce is the prevention of contamination during trimming and washing. Best practices such as general GHP and introducing appropriate cleaning with the addition of sanitizers in water both for cleaning utensils and vegetables should be implemented. Basic hygienic training for workers has to be implemented as well as the routine hygienic inspection from customer, particularly the export company. Temperature should be controlled during transportation to delay growth of bacteria (Kader and Rolle, 2004).

5. Risk characterization

All parameters used to calculate the risk estimate obtained from this study and references which were summarized in Tables 27-28. In order to calculate risk associated with consumption of sweet basil and coriander, the Thai daily consumption data was cited from the National Bureau of Agricultural Commodity and Food Standards (ACFS, 2007). Thai consumption revealed that the intake of sweet basil and coriander per meal are 8.65 and 4.7 g per meal (mean values). The 97.5 % of intake of sweet basil and coriander per meal are 15 and 12 g per meal, respectively. The probabilistic distribution of sweet basil and coriander consumption are RiskLognormAlt("mu",8.65,97.5%,15,"loc",0,RiskTruncate(0,)) and RiskLognormAlt ("mu",4.7,97.5%,12,"loc",0,RiskTruncate(0,)), respectively. Our study assumed that consumers abroad (import country) consume the same amount as Thai consumers.

The probabilistic distribution of *Salmonella* spp., data obtained from Ontoum, (2010) which were surveyed in farm SA and CA during 2009 and enteropathogenic *E. coli* (derived from data of SD farm and CC farm during 2011) to estimate the safety of exported produce.

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Table 27 The summary of variable parameters using in risk estimate (*Salmonella* spp.)

Variable	Description	Distributional assumption	Value		Unit
			Sweet basil	Coriander	
P_i	Probability of illness	P_i = PExP_i(D)			
P_E	Probability of exposure to pathogenic bacteria	P_E = Prevalence×(1-exp $^{(-(C\ xM)}$)			
Prevalence	Prevalence of *Salmonella* contaminated in vegetable		RiskBeta(13,4)	RiskBeta(15,2)	
C[a]	Concentraton of *Salmonella* contaminated in vegetable		RiskLogistic(0.78, 0.25)	RiskLogistic(1.04, 0.21)	log CFU/g
M	Meal size (g)		RiskLognormAlt("mu",8.65,97.5%,15,"loc",0,RiskTruncate(0,))	RiskLognormAlt("mu",4.7,97.5%,12,"loc",0,RiskTruncate(0,))	g
P_i(D)	Probablility of illness from dose: Beta Poisson model	$P_i(D)= 1-(1- D/\beta)^{-\alpha}$			
D	Concentraton of *Salmonella* contaminated in vegetable/meal	D = C×M			log CFU/g
Dose response parameter *Salmonella* (FAO/WHO, 2002)					
β	Beta-Poisson parameter		0.4047	0.4047	
α	Beta-Poisson parameter		5587	5587	

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Table 28 The summary of variable parameters using in risk estimate (*E. coli*)

Variable	Description	Distributional assumption	Value		Unit
			Sweet basil	Coriander	
P_i	Probability of illness	$P_i = PE \times P_i(D)$			
P_E	Probability of exposure to pathogenic bacteria	P_E = Prevalence × (1-exp $^{(-(C \times M))}$)			
Prevalence	Prevalence of *E. coli* contaminated in vegetable		RiskBeta(2,21)	RiskBeta(22,1)	
C	Concentraton of *E. coli* contaminated in vegetable		RiskBetaGeneral(0.08, 0.4, 0, 0.02)	0*	log CFU/g
M	Meal size (g)		RiskLognormAlt("mu",8.65,97.5%,15,"loc",0,RiskTruncate(0,))	RiskLognormAlt("mu",4.7,97.5%,12,"loc",0,RiskTruncate(0,))	g
$P_i(D)$	Probablility of illness from dose: Beta Poisson model	$P_i(D) = 1-(1- D/\beta)^{-\alpha}$			
D	Concentraton of *E. coli* contaminated in vegetable/meal	$D = C \times M$			log CFU/g
Dose response parameter *E. coli* (FSIS, 2001)					
β	Beta-Poisson parameter		0.221	0.221	
α	Beta-Poisson parameter		3,110,000	3,110,000	

*: *E. coli* was not detected in all samples (detection limit was 5 CFU/g)

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In the case of coriander (year 2011), *E. coli* was not detected in exported coriander (detection limit was 5 CFU/g or 0.7 log CFU/g), then we assumed that the population of *E. coli* contamination in exported coriander was 0 log CFU/g.

Lee *et al*. (2009) reported the prevalence pathogenic *E. coli* isolated from fresh beef, poultry and pork in Korea. A total of 39 pathogenic *E. coli* isolates from 273 isolates (3000 samples) were categorized into 3 virulence groups which were enterotoxigenic *E. coli* (43.6%), enterohemorrhagic *E. coli* (35.9%) and enteropathogenic *E. coli* (20.5%). From these results, we assumed that 100% of generic *E. coli* consisted of 14.30% (39/273 isolates) of pathogenic *E. coli* which were 2.93% (8/273 isolated) enteropathogenic *E. coli* (EPEC).

According to FAO/WHO (2002), the enteropathogenic *E. coli* (EPEC) dose response curve was used in this study. In order to present risk as the probability of illness from EPEC contaminated in sweet basil and coriander, the population of generic *E. coli* contaminated in vegetables was converted to enteropathogenic *E. coli*. The average population of generic *E. coli* contamination in sweet basil and coriander were 0.03 and 0 log CFU/g, respectively. Therefore the population of enteropathogenic *E. coli* contamination in sweet basil and coriander were assumed as 0.001 and 0 log CFU/g, respectively (Appendices A). The probability of illness (P_i) at point of consumption was estimated by the combination of the exposure level and dose-response curve and calculated as a Monte Carlo simulation using @Risk™ program (with 10,000 iterations).

5.1 To estimate the probability of illnesses from enteropathogenic *E. coli* and *Salmonella* spp. contamination in sweet basil and coriander.

The probability of illness was summarized as shown in Tables 29-30. In the present, *Salmonella* spp. and *E. coli* dose response data in Thailand are not available, thus dose response model of *Salmonella* spp. (*Salmonella* Naïve) and

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E. coli (EPEC) were used in this study, which are Beta Poisson models, α and β value were 0.4047, 5587 for *Salmonella* spp. and 0.221, 3110000 for *E. coli*, respectively. Both models were estimated by using trial feeding data of human feeding trial of non-typhi *Salmonella* Naïve and surrogate pathogens EPEC/*Shigella* (FAO/WHO, 2002; FSIS, 2001).

According to the estimate of the dose response curve, the individual risk resulting from sweet basil and coriander consumption of 8.65 and 4.7 g/day will expose someone to *Salmonella* spp. at level of 0.57 and 1.43 log MPN/serving size and enteropathogenic *E. coli* at levels of 0.001 and 0 log CFU/g, respectively. It explained the possibilities of *Salmonella* spp. contamination in sweet basil or coriander were 4.88 or 6.70 log MPN/meal, respectively. While the population of enteropathogenic *E. coli* contamination in sweet basil or coriander possible contamination level were 0.03 or 0 log CFU/meal, respectively.

The probabilities of illness per serving when consumed sweet basil or coriander contaminated with *Salmonella* spp. were 37.9 or 31.2 per 100,000 populations, respectively (Table 29). It means that out of every one million consumers to eat food containing sweet basil or coriander for each meal, there will be 379 or 312 consumers possibly made sick from consuming *Salmonella* spp. It could be noted that the illness was calculated from data in 2009. Only 15 samples of each exported vegetables were taken to analyze the population of *Salmonella* spp. The data obtained from 2009 might not represented the situation in 2011 because Thai government strictly controls the export company in 2009-2011 causing the production process to be modified. In addition, Thai fresh produce was detected as *Salmonella* spp. contamination 37tims in 2009 (from Rapid alert system). The alert was deceased to 3 times in 2011 (RASFF, 2011). The concentration of *Salmonella* spp. contamination in exported vegetables in 2009 should be lower than in 2011. The illness in 2011 should be lower than the predicted values in 2009.

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The risk of illness when consuming the sweet basil or coriander contaminated with enteropathogenic *E. coli* were 1.42×10^{-5} or 0 per 100,000 populations, respectively (Table 30). It means that every 100 million consumers to eat food containing sweet basil or coriander for each meal, there will be 0.014 or 0 consumers possibly made sick from consuming enteropathogenic *E. coli*.

However, the consumption data was estimated based on Thai consumption data. The foreigner consumption might lower than Thai people. However some people who consume a lot of fresh vegetables, such as vegetarian, might be ill more than the estimated risk.

6. Variation and uncertainty

Although the numbers of sample taken in this study were 466 samples, collecting from 2 farms, 6 packing houses and 4 factories, but it might not be enough to represent the production process of all Thai exported fresh produce. The difference in practices and management in farm, packing house and factory were attributed to variability in the population and prevalence of *E. coli* in vegetables and in environmental samples since the population of *E. coli* contamination was varying in vegetables. The seasonal variation in 2009 and 2011 might affect the population of *E. coli* contamination in vegetables

In 2011, in order to avoid the EU's stringency inspection, the Thai Agriculture and Cooperatives Ministry opted for a self-imposed ban on 5 groups (16 vegetables). Unfortunately, sweet basil and coriander were included in the 16 vegetable types. Therefore, some vegetable samples collected in 2011 were not processed for export. In addition, Thai government strictly controls the export company causing the production process to be modified. The change in process might affect the population of *E. coli* contamination in vegetables.

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In addition, the pathogenic *E. coli* strain in vegetables was varying. Because the vegetables were cultivated and exposed to an open environment since the farm environment could not be controlled, the contamination pathway in the farms was varied. When calculating the risk estimate, the population of enteropathogenic *E. coli* and its dose response curve are used with the data obtained from literature review. Moreover, the consumption data was used to calculate the serving size of sweet basil and coriander assumed by using Thai consumption data. Then, the risk of illness when consuming the sweet basil or coriander contaminated with enteropathogenic *E. coli* might be lower or overestimated.

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Table 29 Probability of illnesses estimated when consumeing the sweet basil and coriander contaminated with *Salmonella* spp. time of consumption

Types	P_i*	P_i*/100,000 meal	Probabilistic distribution
Sweet basil	1.46×10^{-10}	1.46×10^{-5}	Distribution for E basil/F23
Coriander	0	0	-

*: P_i is the probability of illness

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Table 30 Probability of illnesses estimated when consumeing the sweet basil and coriander contaminated with *E. coli* at time of consumption

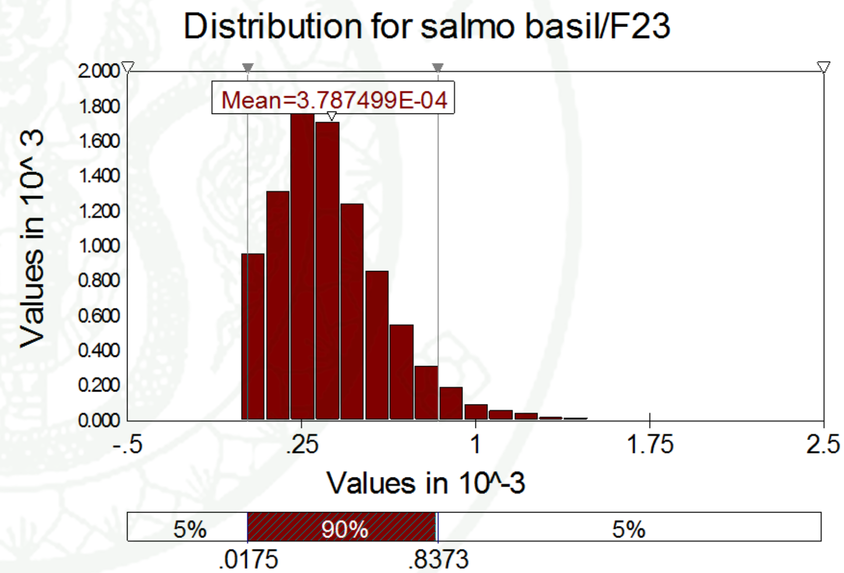
Types	P_i*	P_i*/100,000 meal	Probabilistic distribution
Sweet basil	3.79×10^{-4}	37.9	

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Table 30 (Continued)

Types	P_i*	P_i*/100,000 meal	Probabilistic distribution
Coriander	3.12×10^{-4}	31.2	Distribution for Salmo coriander/F23 Mean=3.124914E-04 Values in 10^ 3 Values in 10^-3 5% 90% 5% .0698 .7431

*: P_i is the probability of illness

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7. Recommendation of control measure to prevent pathogenic bacteria contamination in fresh produce for export

Control measures for a variety of harvesting practices would decrease the risk of pathogenic bacteria contamination Although for different fresh produce the control steps would be different, key factors are field worker hygiene, field sanitation, equipment sanitation, and temperature control.

Farm

Manure: the fully composted manure should be applied in the farm. The manure should be stored in a closed environment to avoid the recontamination from humans, pets, and dust. During compost process, the manure should be turned and mixed to maintain the core temperature of (>55°C) and only composted manure allowed to use in the farm.

Irrigation water: drip irrigation systems with ground water should be applied in farm in order to reduce the chance of soil spreading when watering. It could be noted that water system and water source should be considered.

Farm facilities: basic facilities such as toilets, hand washing facilities, and protective clothing should be provided for the farmers.

Packing house

Personal hygiene, cleaning of utensil and wash water poss the potential risk factors which are all related to the quality and safety of vegetables. Proper cleaning procedure with sanitizing agent can reduce the risk of pathogenic contamination on vegetables. Sanitary conditions of farm environment are more difficult to maintain compared with packing house environment. Proper controlling temperature and humidity assists to keep quality and shelf life of vegetables, and prevent growth of microorganisms particularly pathogen of our concern, *E. coli* and *Salmonella* spp.

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Lastly training and simply education for farmers and workers are the crucial of preventive measure to be considered in order to enhance food safety aspect in fresh produce for consumption both domestic and international market.

Personal hygiene: hands are a very common vehicle for the transfer of human pathogens to food products. Hands may become contaminated when touching dirty vegetables. The microbial contamination in dirty vegetables could transfer to clean vegetables. Moreover, a hand washing method using sanitizer during process should be promoted to workers for reducing cross contamination.

Utensils: maintain a clean environment in the packing house to limit the chance of cross contamination from humans, dust, insects, and pets by using things such as window screens. The utensils used in trimming step should be cleaned with sanitizer before, during, and after the process. Moreover, baskets and cover material should be clean as well. Cleaning the baskets and utensils could accomplish by hand sweeping or brushing in order to remove the dust on the baskets and utensils.

Wash water: the leafy vegetables and herbs can be considered a ready to eat food. It is important to use water of potable quality in the washing step at the packing house. During the washing step, the cross contamination could occur. Chlorine based sanitizer or the proper sanitizer should be added to the wash water maintaining the circulating water system to inhibit/reduce the cross contamination during the washing process.

Transportation: temperature is an important factor in maintaining post harvest quality. The temperature should be controlled during transportation in order to prevent microbial growth and maintain the vegetables' quality. During transportation, the vegetables should be packed or carried in a closed system to prevent cross contamination from dust, humans and insects.

Training and education system: it is important to evaluate the training and education programs targeted to the farmers and workers along the entire vegetable

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production chain such as farm, packing house, and factory. It should be considered as a primary preventative control measure. The training program, such as GAP, GHP and GMP, should be educated to worker. The main focus of a training program is improving awareness, knowledge, and worker skill.

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CONCLUSIONS

From the experimental results and discussion of this study, the conclusion can be drawn as follows

There were differences in the distribution of population of *E. coli* contamination in fresh produce samples and environmental samples from different farms and packing houses. In the farm environment, *E. coli* was detected in all sources of farm environment (seed, soil, fertilizer and irrigation water). Soil and irrigation water might be the sources of contamination in the pre-harvest environment, particularly water sources from furrow with surface water which are used in sweet basil farms. Water reservoir impacts on the level of *E. coli* contamination since irrigation water used in coriander farms comes from underground water, which is expected to be less contaminated.

E. coli contamination was found on utensil samples (table, gloves or bare hand, scissors, baskets, and cover material) used in the packing houses; both the basil farm and coriander farm used improper cleaning and sanitizing. It was observed that the wash water had not been changed during the washing process. It was contaminated with a heavy load of *E. coli,* indicating a poor quality of water. Detection of *E. coli* in the environmental samples at the packing houses indicates a need for improve cleaning and sanitation protocols to reduce pathogenic bacteria from being transferred to the final product.

The prevalence of *E. coli* contamination in sweet basil and coriander trended to be increased throughout the process. Then population of *E. coli* contamination in vegetables after trimming was higher than after harvesting. Moreover, the population of *E. coli* contamination in vegetables after washing was higher than before washing. We recommended that the vegetables should not washed before distribution to factory; or if the washing process is needed, the sanitizing agent should be applied to prevent the cross contamination.

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From our results, the population and prevalence of *E. coli* contamination in exported sweet basil and coriander in 2009 was higher than in 2011 because the production process was modified with strictly controls. Moreover, *E. coli* O157:H7 was not detected in the exported sweet basil and coriander sample in 2011.

Cross contamination during the washing process could occur when simultaneously washing the sound vegetables and inoculated vegetables or washing in reused water. Chlorinated water (50 ppm of available chlorine) assisted to inhibit the cross contamination during the washing process even though the reused water was applied in the washing process.

The sensitivity analysis of simple approach model showed the population of *E. coli* contamination in sweet basil and coriander after harvesting was the greatest factor affecting the population of *E. coli* contamination in fresh produce at receiving factory. Moreover, the difference of *E. coli* population during the handling and trimming processes were a big factor to affect the population of *E. coli* in fresh produce at the receiving factory.

The result of sensitivity analysis of liner regression models showed the population of *E. coli* contamination in sweet basil after washing and after harvesting had the greatest impact on the population of *E. coli* contamination in vegetables at the receiving factory. On the other hand, the population of *E. coli* contaminated in coriander after trimming showed a big impact, influencing the population of *E. coli* at the receiving factory. Because the spreading of *E. coli* from soil to vegetables was occurred during trimming process.

The individual risk resulting from sweet basil and coriander consumption of 8.65 and 4.7 g/day will expose the consumer to *Salmonella* spp. at a level of 0.57 and 1.43 MPN/g and enteropathogenic *E. coli* at levels of .001 and 0 log CFU/g, respectively. The risk of illness per a 100,000 population when consuming the sweet basil or coriander contaminated with *Salmonella* spp. were 37.9 or 31.2 per 100,000 consumers, respectively. It means that out of every one million consumers that eat

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food containing sweet basil or coriander for each meal, there will be 379 or 312 consumers possibly made sick from consuming *Salmonella* spp. It could be noted that the data used to estimate the illness was in 2009. While the risk of illness when consuming sweet basil or coriander contaminated with enteropathogenic *E. coli* were 1.42×10^{-5} or 0 per 100,000 consumers, respectively. It means that out of every 100 million consumers that eat food containing sweet basil or coriander for each meal, there will be 0.014 or 0 consumers possibly made sick from consuming enteropathogenic *E. coli*.

The recommendation for the fresh produce export industry is that serious GAPs and GHPs need to be implemented at farms and packing houses. The suggested interventions were to prevent cross contamination during the washing process. Moreover, utensils/facilities need to be routinely cleaned with sanitizing agent.

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APPENDICES

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Appendix Table A1 Transfer rate of *Enterobactor aerogenes* from contaminated sweet basil to water and water to sound sweet basil

Sequence of washing	CFU/g,ml			Transfer rate (%)		Log % transfer rate	
	Contaminated vegetables	Sound vegetable	Wash water	Twv_s [a]	Tv_cw [b]	Twv_s [a]	Tv_cw [b]
Initial load	15850	-	-	-	-	-	-
	8710	-	-	-	-	-	-
	19060	-	-	-	-	-	-
1st washing	6460	-	1180	-	7.4	-	0.9
	1740	-	910	-	10.5	-	1.0
	3630	-	760	-	3.9	-	0.6
2nd washing	-	350	1550	30.0	-	1.5	-
	-	809	870	8.70	-	0.9	-
	-	180	780	24	-	1.4	-
3rd washing	-	460	1450	29.5	-	1.5	-
	-	140	870	16	-	1.2	-
	-	100	850	13.0	-	1.1	-
4th washing	-	650	1590	44.9	-	1.7	-
	-	210	1230	24.3	-	1.4	-
	-	390	600	45.4	-	1.7	-

[a]: Twv_s is the transfer rate of *E. aerogenes* from wash water to sound vegetable

[b]: Tv_cw is the transfer rate of *E. aerogenes* from contaminated vegetable to wash water

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Appendix Table A2 Transfer rate of *Enterobactor aerogenes* from contaminated coriander to water and water to sound coriander

Sequence of washing	CFU/g,ml			Transfer rate (%)		Log % transfer rate	
	Contaminated vegetables	Sound vegetable	Wash water	Twv_s[a]	Tv_cw[b]	Twv_s[a]	Tv_cw[b]
Initial load	22910	-	-	-	-	-	-
	23990	-	-	-	-	-	-
	12590	-	-	-	-	-	-
1st washing	4680	-	1100	-	4.8	-	0.7
	5500	-	1290	-	5.5	-	0.7
	2820	-	1020	-	8.1	-	0.9
2nd washing	-	250	1100	23.0	-	1.4	-
	-	350	1150	26.9	-	1.4	-
	-	100	1150	9.8	-	1.0	-
3rd washing	-	250	1180	23.0	-	1.4	-
	-	150	1020	13.0	-	1.1	-
	-	150	1000	13.1	-	1.1	-
4th washing	-	550	810	46.4	-	1.7	-
	-	450	1510	43.5	-	1.7	-
	-	250	1100	25.0	-	1.4	-

[a]: Twv_s is the transfer rate of *E. aerogenes* from wash water to sound vegetable

[b]: Tv_cw is the transfer rate of *E. aerogenes* from contaminated vegetable to wash water

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Appendix Table A3 The population and probabilistic distribution of enteropathogenic *E. coli* contamination in exported sweet basil and local coriander

Types	Sweet basil	Coriander
Generic *E. coli* (logCFU/g)	0.03	0
Enteropathogenic *E. coli* (EPEC, logCFU/g)*	0.001	0
Probabilistic distribution	RiskBetaGeneral(0.08, 0.40, 0, 0.15)	-
		-

*: The population of EPEC in coriander was calculated from Lee *et al.* (2009)

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