Characterizing food safety aspects of the Cambodian vegetable value-chain: A quantitative and qualitative investigation of biological hazards and food safety practices in Cambodia

by

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B.S., Purdue University, 2017

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

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Abstract

Food safety is a major challenge in low and middle-income countries (LMIC). In Cambodia, diarrheal disease is a prominent cause of childhood mortality. Recent findings suggest that greater than 40% of diarrheal diseases in developing nations are attributed to contaminated food. The majority of consumers in Cambodia purchase food through informal markets that escape food safety standards and controls. This is primarily due to the fact that food safety regulations are often poorly enforced, and infrastructural and educational capacity for food safety is limited, particularly in informal market settings. This is critical, since a majority of consumers in LMIC purchase vegetables that are often consumed raw through informal markets. The consumption of contaminated raw vegetables is heavily linked to foodborne illness, and likely contributes to morbidity and mortality in the country. Currently, little data exists on the biological contamination (i.e. bacteria, viruses, and parasites) of vegetables being sold in informal markets in Cambodia.

This research seeks to investigate "What is the prevalence and concentration of bacterial hazards on vegetables sold in Cambodian informal markets, and how do handling practices, from farm- to-market, contribute to food safety outcomes in the vegetable value-chain of Cambodia?". Based upon this, the following objectives were chosen: (1) to investigate the prevalence and concentration of *Salmonella enterica*, as well as the concentration of generic *Escherichia coli* (*E. coli)* and coliforms, on different types of fresh vegetables sold in Cambodian informal markets in different seasons; (2) to define the flow and the behaviors of stakeholders within the Cambodian vegetable value-chain through personal interviews; and (3) to identify practices that can potentially contribute to the cross-contamination of vegetables moving through the value-chain. It was hypothesized that the prevalence and concentration of bacterial hazards would vary by vegetable type and seasons, due to differences in food matrix and growth conditions, as supported by

scientific literature. Furthermore, it was hypothesized that due to limited educational, physical and regulatory capacity, the likelihood of poor handling practices by vegetable value-chain actors would be high, and contribute to contamination of vegetables along the value chain.

Three types of vegetables (lettuce, tomatoes, cucumbers) were collected from informal markets located in two different provinces (Battambang and Siem Reap) in Cambodia in two different seasons (rainy and dry). Samples were subjected to validated methods for the detection of *Salmonella enterica*, and the enumeration of *Salmonella enterica*, generic *E. coli,* and coliforms. Presumptive positive isolates of *Salmonella enterica* were confirmed using Real-Time PCR. Further, a survey tool was used to investigate the flow of the Cambodian vegetable value-chain, and personal interviews with value-chain actors was used to characterize food safety practices within this value-chain (i.e. farmer, collector, distributor and vendor).

The highest prevalence of *Salmonella enterica* was found on lettuce collected in the dry season (55.8%), whereas the lowest prevalence was found on lettuce collected in the rainy season (15.4%). In terms of concentration of *Salmonella enterica*, lettuce had a significantly higher concentration (5.66 $\log_{10}$ CFU/g), as compared to cucumbers (4.20 $\log_{10}$ CFU/g) and tomatoes (3.99 log CFU/g). Further, vegetables in the rainy season (5.27 $\log_{10}$ CFU/g) had significantly higher counts of *Salmonella enterica* as compared to vegetables in the dry season (3.96 log CFU/g). Moreover, the highest concentration of *E. coli* and coliforms were observed on lettuce during the rainy season (2.75 and 6.31 $\log_{10}$ CFU/g respectively). Conversely, the lowest concentration of *E. coli* and coliforms were observed on cucumbers (0.81 $\log_{10}$ CFU/g) and tomatoes (3.89 $\log_{10}$ CFU/g) collected in the dry season. Survey results support that a high percentage of the value-chain actors do not practice cool storage, which increases the likelihood of microbial proliferation as well as decrease the quality of vegetables. Additionally, the wide use

of inadequately composted animal-source waste, contaminated irrigation water, and the lack of basic food hygiene practices (e.g. using fabric gloves, selling raw meat in the same area as fresh vegetables, lack of handwashing practices etc.) were factors that may be contributing to the introduction of pathogens through cross-contamination.

Findings from this study highlighted that vegetables sold in informal markets in Cambodia are contaminated with biological hazards, with high concentrations being observed across all vegetable types and seasons. Contaminated vegetables can introduce bacterial pathogens into informal markets. Furthermore, contamination may be increased through the lack of basic food hygiene practices of value-chain actors, particularly informal market vendors. Many of these practices promote cross-contamination of bacteria from the environment to the vegetables. For these reasons, interventions such as food safety recommendations in the form of training and education programs for value-chain actors, regulatory coordination, consumer communication programs and infrastructure development are necessary to reduce the likelihood of contamination and negative public health outcomes in the country.

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Acknowledgements

I would like to thank my major advisor, Dr. Jessie Vipham, for her knowledge and guidance throughout my academic journey. Thank you for giving me the opportunity to explore my passion for food safety in developing countries setting. I would also like to thank Dr. Randall Phebus and Dr. Londa Nwadike for your willingness to participate in my committee and for your advice in my research and thesis.

I would like to thank Carla Schwan; your presence and mentorship motivated me to complete this degree. I could not have imagined sharing this two-year journey with someone other than you. I would also like to thank Dr. Valentina Trinetta and Dr. Sara Gragg their advice and generosity in letting us use their laboratory space and equipment. Further, I would like to thank the food science graduate students for your help during my day-to-day life in Call Hall and for creating an enjoyable working environment.

I would like to thank the faculty members and students at the Royal University of Agriculture, Cambodia. Thank you to Dr. Lyda Hok, Dr. Sath Keo, Bunna Chea, and Kroesna Nita for your expertise and generosity. Thank you to Visoth, Malyheng, Chhunna, Emmy, Dara, Sreyroth, Chun, and other students for your enthusiasm and help in data collection.

I would like to express my heartfelt gratitude to my family members in Indonesia, especially my mom, Riesa and my grandparents, Josie and Frans. Your constant love and support gave me the strength to keep fighting for this degree. Thank you to Derico, for believing in my abilities and for continuing to lift me up during the hardest moments. I would also like to thank Zsazsa and my closest friends for their kind encouragement.

Finally, I would like to acknowledge Feed the Future and United States Agency for International Development for making this research possible.

Chapter 1 - Literature Review

1.1 Global burden of foodborne illness

It is hypothesized that foodborne illness is a persistent challenge for public health, economic growth, international trade, and agricultural development in several low-and middle-income countries (LMIC). Evidence shows that unsafe food significantly increases risk of morbidity and mortality of a population, especially children below five years at age (Grace, 2015). Furthermore, according to Guerrant, DeBoer, Moore, Scharf, and Lima (2013) diarrheal disease is responsible for stunting in one-fifth of the world's children, with a majority of this burden occurring in developing nations. In fact, diarrheal diseases in the early years of childhood negatively impact cognitive development of children and expose them to the increased risk of obesity and non-communicable diseases (Guerrant et al., 2013). Food safety also drives down the economy of LMIC as it can decrease trading confidence, leading to a reduction in export from failure to meet international standards (Grace, 2015). Furthermore, unsafe food can lead to financial losses from income loss and medical expenses (Grace, 2015; Jaffee, Henson, Unnevehr, Grace, & Cassou, 2019). A recent study by the World Bank estimates that around $110 billion in productivity and medical expenses are lost each year from unsafe food, in LMIC. Most of these costs were accrued from South Asia, Southeast Asia and Sub-Saharan Africa. These countries account for 41% of the global population, but are afflicted with 53% of all foodborne illness and 75% of related deaths (Jaffee et al., 2019).

Foodborne illness also has detrimental impacts on women's health and livelihoods. Pregnant and lactating women are vulnerable to a wide range of foodborne diseases. Moreover women play an important —sometimes dominant—role in many traditional food chain, especially at retail. Grace (2015) reported that attempts to modernize food value-chain to address food safety

threats, have often lead to the exclusion of women in the agriculture value-chain. Findings observed that many modern, private poultry sector in South Africa only employ male workers, and that men have predominate the marketing of peri-urban dairying sector of West Africa (Roesel & Grace, 2014). Overall, the impact of food safety has greatly extended beyond the area of public health, but also significantly impacting the economic productivity and social roles of a country. Despite the alarming findings, much is currently unknown about the full burden of foodborne hazards, particularly in developing nations. It was not until 2006 that the World Health Organization (WHO) instigated a consultation, attended by international experts, to study the cost of unsafe foods on a global level. This consultation resulted in the establishment of the Foodborne Disease Burden Epidemiology Reference Group (FERG) (Havelaar et al., 2015).

The FERG initiated a global study in order to quantify the burden of foodborne disease using a hazard and incidence-based approach (Havelaar et al., 2015). The FERG used a common epidemiological equation, Disability Adjusted Life Years (DALY), to measure the global burden; which represents the number of life years lost due to illness, disability or death (Devleesschauwer et al., 2014). DALY is calculated by adding its components: years of life lost to mortality (YLL) and the number of years lived with disability due to morbidity (YLD). A source attribution tool and results from existing scientific literatures were also used to attribute enteric disease to its major transmission route (e.g. food, water, animal reservoir etc.). These findings were compiled and published as the 2015 WHO Estimates of the Global Burden of Foodborne Diseases. This report was the first of its kind, and estimated that 600 million cases of foodborne illness occur annually, and that the burden of foodborne disease is similar to those of HIV, malaria and tuberculosis (Havelaar et al., 2015). These estimates amplify the significance of addressing foodborne pathogens as real threats to public health.

The 2015 WHO report organized and presented its findings by sub-regions. As illustrated in Figure 1.1, all countries were segregated into a total of 6 sub-regions: Africa (AFR E & D), America (AMR A, B & D), Eastern Mediterranean (EMR B & D), Europe (EUR A, B & C), Southeast Asia (SEAR B & D) and the Western Pacific (WPR A & B). Each letter associated with the sub-regions represents the level of child and adult mortality; stratum A = very low child and adult mortality, stratum B=low child mortality and very low adult mortality, stratum C=low child mortality and high adult mortality, stratum D= high child and adult mortality and stratum E = high child mortality and very high adult mortality (Havelaar et al., 2015).

Overall findings of the WHO report showed that the burden of foodborne disease reached a staggering 33 million DALY in 2015, with one out of ten people contracting foodborne illness each year. Findings also showed that children bear the highest burden from foodborne illness, with one third of the total deaths from foodborne disease being children under five. On a sub-region level, the AFR D and E regions carried the highest burden with 1,300 and 1,100 DALY per 100,000 population, respectively, followed by SEAR B and D with 690 and 710 DALY per 100,000 population respectively (Havelaar et al., 2015). The WPR region was all in the range of 40-50 DALYs per 100,000 population. Among foodborne illness cases caused by various hazards (i.e. biological, physical, chemical), the highest number of illnesses were attributed to biological hazards, such as bacteria, viruses and parasites. Virus caused the highest number of illnesses, but bacteria caused the highest number of deaths compared to other biological agents (Havelaar et al., 2015).

The WHO data provided new insights on the global burden of foodborne disease and its hazards. However, the report listed a few constraints, specifically the lack of surveillance data and existing literature on foodborne disease and hazards in developing nations. As a result, the

quantification of foodborne disease burden for these countries was solely based on structured elicitation and scientific judgement from experts, instead of published evidence (Havelaar et al., 2015). This emphasized the need for further research on foodborne hazards in developing nations, such as Southeast Asia, in order to fully capture the risk of foodborne disease in the nation. Moreover, research concerning bacterial agents need to be further explored, as infections by bacteria has caused the highest mortality compared to other agents.

1.2 Global and national public health outcomes

Public health outcomes heavily influence a nation's ability to develop, both physically and economically. Productive societies are based upon productive individuals, all of which can be heavily influenced by the *public health environment* for a given society (Figure 1.2). Food safety is one aspect of public health that needs to be more deeply explored and addressed in LMIC. Developing a strong understanding of food safety's role in agriculture value-chain development is a critical step to ensuring that national and global public health outcomes are achieved. This includes understanding the sources and transmission of foodborne hazard contamination (i.e., biological, chemical, and physical) within agriculture *value-chain environments*; the role of risk (i.e., probability and severity of disease) in linking foodborne hazards to the *public health environment*; defining the influential factors that contribute to public health and development outcomes (e.g., economic factors, political factors, social factors, etc.); and investigating the interactions between individual development outcomes, in the *public health environment* (Figure 1.2).

1.2.1 Public health outcomes in Southeast Asia

Southeast Asia, a region consisting of eleven countries, is home to half a billion of the world's population (Chongsuvivatwong et al., 2011). This region has immense social, economic and political diversity across and within countries. Southeast Asia has become one of the fastest growing region of nations in terms of economic expansion in the world (Suk et al., 2003). However, this high rate of economic success has not been coupled with equivalent improvements in the public health sector. Currently, Southeast Asia hosts the highest child mortality rate in the world; one-third of the world's estimated 1.7 million child mortalities, for children under five years of age, occurs in Southeast Asia (Suk et al., 2003). Furthermore, nine out of ten of these deaths are attributed to infectious diarrhea (Suk et al., 2003). Historically, unsafe water and poor sanitation were considered the prominent causes of infectious diarrheal disease, however, a recent report by the World Health Organization (2015) estimated that 29% of enteric diseases occurring in the world are caused by contaminated food (Havelaar et al., 2015). Although data exist on the global burden of foodborne disease, limited information remains on a country-specific level, especially in developing nations, such as Cambodia.

1.2.2 Cambodia

Bordered by Thailand in the Northwest and Vietnam in the Southeast, Cambodia is a developing nation in Southeast Asia with land area of 181,035 square kilometers (National Institute of Statistics Cambodia & Directorate General for Health, 2015). In 2014, its population reached 15.4 million people, with 80% of the total population living in the rural areas of the country and depending on farming as their main source of income (Food and Agriculture Organization of the United Nations, 2015). Despite major agricultural and economic growth in the last years, Cambodia has experienced several setbacks in the past few decades, making it one of the least

developed nations in Southeast Asia. Perhaps most notably, the genocidal regime of the Khmer Rouge in the 1970s negatively affected Cambodia's growth, and ultimately its current socio-economic standing in the world. It is estimated that, during the rule of the Khmer Rouge, nearly 2 million people were killed, mainly those with intellectual professions, and the stock market and public schools were closed (National Institute of Statistics Cambodia & Directorate General for Health, 2015). Most urban Cambodians were forced to work as agricultural laborers and by the end of the third year of the Khmer Rouge, one out of four Cambodians died of overwork, malnutrition, misdiagnosed disease or execution (Chandler, 2009). This unfortunate event caused a setback for Cambodia's economy and obliterated all efforts made toward education and literacy in the previous two decades. As a result, a fraction of the country lacks the educational and infrastructural capacity to address several public health challenges, especially food safety issues that occur within their daily lives.

1.3 *Salmonella enterica*

Salmonella is a rod-shaped Gram-negative enteric bacterium, which is an important food pathogen in developed and developing nations (Akhtar, Sarker, & Hossain, 2014). It is a facultative intracellular anaerobe that belongs to the Enterobactericeae family. There are two species of *Salmonella*, namely *Salmonella enterica* and *Salmonella bongori*. However, only the species *enterica* is commonly discovered in clinical samples and cause diseases in humans. *Salmonella enterica* has six subspecies including enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI). These subspecies are further classified into serovars, which are differentiated by their flaggelar, carbohydrate and lipopolysaccharide (LPS) structures. Over 2,500

serovars of *S. enterica* have been identified, but a majority of serovars known to cause disease in humans belong to subspecies enterica (I) (Coburn, Grassl, & Finlay, 2007).

S. enterica serovars are transmitted orally and can cause distinct clinical symptoms: (1) typhoid fever, which is caused by *S. enterica* subsp. *enterica* serovar Typhi; (2) gastroenteritis, which is caused by non-typhoidal *Salmonella* serovars in immunocompromised individuals and (3) bacteraemia, which is caused by non-typhoidal *Salmonella* serovars in immunocompromised individuals (Keestra-Gounder, Tsolis, & Bäumler, 2015). Both *Salmonella* Typhi and Non-typhoidal *Salmonella* can be transmitted through contaminated food, and symptoms often occur between 6 to 72 hours after consumption (Coburn et al., 2007). Non-typhoidal *Salmonella* causes the highest number of food related deaths globally with over 59,000 estimated deaths in 2010, and creating the largest global burden among other foodborne disease agents (Havelaar et al., 2015).

1.3.1 *Salmonella enterica* virulence factors

There are numerous studies investigating the bacterial pathogenesis of *Salmonella enterica*. In vitro tissue culture and small animal models are frequently used to investigate the complex interactions between host and pathogen during infections (Coburn et al., 2007). Although these models do not necessarily reflect human infection, they give some insights on how *Salmonella enterica* invades, resists and manipulates the host's immune system for its survival.

A significant component that determines *Salmonella enterica*'s ability to stimulate infection is in its genetic structure. Analysis of the genetic material of the bacterium revealed that virulence genes are clustered in localized regions of the chromosome, called the *Salmonella enterica* Pathogenicity Island (SPI) (Groisman & Ochman, 1996). It was discovered in 1996 that these SPIs are horizontally transferred from non-pathogenic bacteria. There are 14 SPIs known to date and

each serovar of *Salmonella enterica* has a distinct number of islands. *Salmonella* Typhimirium, for example, has at least five SPIs in its genome (Rhen, 2007). SPI-1 and SPI-2 are the two most studied islands due to their role in the Type III Secretion System (T3SS), a mechanism to deliver virulence proteins termed effectors into the host cell cytoplasm (Hensel et al., 1998). Effectors alter basic host-cell functions such as signal transduction, cytoskeletal formation, membrane trafficking and chemokine gene expression to the advantage of the bacterium (Ohl & Miller, 2001). Another important virulence mechanism is *Salmonella enterica*'s two-component regulatory system that regulates gene expression in response to environmental cues (Ohl & Miller, 2001). The controlled up-regulation and down-regulation of some genes alter *Salmonella*'s physiology and prolongs *Salmonella*'s survival in harsh environmental conditions.

1.3.2 *Salmonella enterica* invasion, colonization and survival

Salmonella enterica infection begins with the ingestion of contaminated food or water. Upon ingestion, the low pH of gastric juices in the stomach serves as the initial barrier to infection. However, studies support that the acid tolerant characteristic of *Salmonella enterica* enable it to survive the acidic environment of the stomach (Garcia-del Portillo, Foster, & Finlay, 1993). Upon colonization of the intestine, *Salmonella enterica* localizes to the apical epithelium and internalizes itself by inducing membrane ruffling that encapsulates the bacteria into large vesicles in the intestinal cell termed *Salmonella*-containing vacuole (Ohl & Miller, 2001). The *Salmonella*-containing vacuole enables the bacteria to survive and replicate within the host cell. This internalization process is governed by effector proteins expressed by SP-1 and translocated using T3SS, that activate intracellular signaling cascades and cytoskeletal rearrangements (Coburn et al., 2007). Specifically, these effector proteins are SopE, which induces the membrane-ruffling

response and SptP, which recovers the membrane form after an invasion. Other effector proteins, SipA and SipC, act on cytoskeletal actin rearrangement to help the entry of *Salmonella enterica* to the intestinal cell (Coburn et al., 2007).

Following internalization, *Salmonella enterica* serotypes that cause enteric diseases such as diarrhea commonly recruit neutrophils to the intestinal tissue by the secretion of chemokine interleukin-8, signifying the beginning of an infection (Ohl & Miller, 2001). *Salmonella enterica* serotypes that cause typhoid fever must survive and replicate within the host macrophage to establish virulence and cause systemic infection (Fields, Swanson, Haidaris, & Heffron, 1986). The macrophage is a harsh environment, as it contains antimicrobial compounds such as reactive oxygen and nitrogen species, antimicrobial peptides and hydrolytic enzymes (Ohl & Miller, 2001). *Salmonella enterica* uses several mechanisms to minimize the effect of these microbicidal compounds. *Salmonella enterica* uses its two-component regulatory system to modify its protein and lipopolysaccharide components of the inner and outer membrane to build resistance to antimicrobial peptides and to produce a membrane with lower inflammatory potential (Ohl & Miller, 2001). A second *Salmonella enterica* T3SS located in SP-2 also secretes effector proteins that prevent phagosome-lysosome fusion and allows the *Salmonella*-containing vacuole to avoid these microbicidal compounds and continue replicating (Uchiya et al., 1999). In addition, *Salmonella enterica* also secretes several enzymes such as homocysteine and superoxide dismutase that directly counteract reactive oxygen and nitrogen species in the macrophage (De Groote, Testerman, Xu, Stauffer, & Fang, 1996) (Fang et al., 1999). These mechanisms play critical roles in *Salmonella enterica*'s resistance to host immunity and ability to establish systemic infection.

1.3.3 Recent *Salmonella enterica* outbreaks

Historically, *Salmonella enterica* was thought to be a concern solely for poultry, eggs, meat products and produce (Centers for Disease Control and Prevention, 2018b). The 2012 multistate outbreak in the United States, due to *Salmonella enterica* Heidelberg in Foster Farm brand chicken, remains a landmark outbreak. A total of 634 people were infected by a multidrug-resistant strain of the pathogen, initiating discussions about the need for stronger regulations in the United States (Centers for Disease Control and Prevention, 2018b). However, recent outbreaks are redirecting the focus on *Salmonella enterica* to other products, or environments that historically have not supported the growth of the pathogen, such as high sugar, low water activity products (Finn, Condell, McClure, Amézquita, & Fanning, 2013). For example, the 2009 multistate outbreak of *Salmonella enterica* Typhimirium in peanut butter, the 2018 outbreak in Honey Smacks cereal and several outbreaks of *Salmonella enterica* in imported spices, enforced the fact that *Salmonella enterica* can survive far beyond its conventional food environments (Centers for Disease Control and Prevention, 2018b). These recent findings have raised the significance of *Salmonella enterica* as a major pathogen of concern due to its ubiquity in food and its ability to cause high numbers of infections.

In Cambodia, outbreak data is not available, however, there is evidence that meat and milk products sold in the informal markets have historically been contaminated with pathogens such as *Escherichia coli*, *Salmonella enterica* and tapeworm cysts (Roesel & Grace, 2014). This can introduce cross-contamination to other food products sold in the markets (e.g. vegetables, cooked food). Moreover, the lack of hygiene practices and infrastructure in the informal markets are likely to increase contamination, as there is growing evidence that *Salmonella enterica* are able to survive in non-food environments (Soumet et al., 1999). Therefore, the ubiquity of *Salmonella enterica* as

well as the conditions in the informal markets of Cambodia, increase the likelihood of cross-contamination of vegetables, which might expose the consumers to more contaminated products.

1.3.4 *Salmonella enterica* epidemiology and surveillance

Epidemiology is the study of distribution (e.g. frequency) and determinants (e.g. causes and risk factors) of health-related events in a specified population (Centers for Disease Control and Prevention, 2016). In food safety, epidemiological data is used to assess the nature of foodborne diseases and to determine strategies to control them. Epidemiologists use various methods to collect epidemiological data in the food safety sector, such as outbreak surveillance to study the distribution of foodborne disease. They also used different analytical measures to study the determinants of foodborne disease, particularly in calculating risk factors. The most common is by analyzing the probability of exposure, severity of the disease and the host factors. *Salmonella enterica* has over 2,500 serovars, each associated with varying levels of risk in causing human disease (Coburn et al., 2007). For this reason, infection by *Salmonella enterica* serovars can be either self-limiting or life threatening, depending on the serovar contracted (e.g. strains of *Salmonella* serotype Heidelberg are commonly multidrug resistant (Coburn et al., 2007; Nair, Vazhakkattu Thomas, Noll, Porter Jr, & Kollanoor Johny, 2018). Serovar type also determines the frequency of exposure, as some serovars are more likely to be found in multiple food sources than in a single food type. Thus, it can be supported that identifying the serovars of *Salmonella enterica* is crucial in determining the risk of disease and predicting the health outcome of a *Salmonella enterica* infection within a human host.

Currently, data regarding *Salmonella enterica* serovars, involved in outbreaks in multiple developed countries are available from the national surveillance bodies (i.e. FSIS, CDC, ECDC).

In the United States, *Salmonella enterica* serotypes Typhimirium, Enteritidis, Heidelberg, and Newport are the serovars that are highly notorious for causing illness (National Center for Emerging and Zoonotic Infectious Diseases, 2013). Conversely, in the EU, *Salmonella enterica* serotypes Enteritidis, Typhimirium, Infantis and Stanley are the most commonly reported *Salmonella enterica* serovars in clinical infections (European Centre for Disease Prevention and Control, 2014). This surveillance data emphasizes that there are major differences in serovars that cause human disease between nations. Unfortunately, surveillance data is limited and does not currently exist in many developing nations, specifically Cambodia. Therefore, it is critical that evaluation of *Salmonella enterica* on a serovar level must be done in Cambodia, to fully understand the level of public health risk that *Salmonella enterica* has presents in the country's food system.

1.4 *Escherichia coli*

Escherichia coli (E. coli) is a facultative anaerobe, gram-negative, rod-shaped bacterium from the Enterobacteriaceae family. *E.coli* is predominantly found in the intestines of humans as part of the gut flora and is a member of the coliform group (Nataro & Kaper, 1998). Although most *E. coli* strains are nonpathogenic, some can be deadly as it has the potential to cause gastrointestinal diseases, sepsis, meningitis and urinary tract infection, especially in children below five and immunocompromised individuals (Nataro & Kaper, 1998). *E. coli* can be categorized based on their O and H antigens. The O:H antigen serotypes, virulence factors and clinical syndromes characterize *E.coli* into six groups: Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Enteroaggregative *E.coli* (EAEC), Diffusively Adherent *E.coli* (DAEC) and Shiga-Toxin-Producing *E.coli* (STEC) (Meng, LeJeune, Zhao, & Doyle, 2013; Nataro & Kaper, 1998). Each of these groups has different features and mechanisms of disease.

For example, ETEC causes traveler's diarrhea in the developing world and EIEC is known to have a similar pathogenesis as *Shigella* (Nataro & Kaper, 1998). However, the group contributing the highest mortality rate and risk of death for children under five in the United States is STEC (Nataro & Kaper, 1998). Over the years, an abundance of research has been conducted in the United States and elsewhere to develop measures to detect and control STEC within the food chain. Unfortunately, this is not the case in most developing nations as data on STEC prevalence are limited in scope and its presence in the food chain is seldom addressed. Therefore, research on STEC in these nations might be an important starting platform in exploring its presence and mitigating its contamination in developing nations.

1.4.1 STEC

STEC is a group of pathogenic *E. coli* that produces cytotoxic compounds called shiga-toxin. Various STEC strains with a broad range of O:H serotypes are capable of causing a wide spectrum of human diseases, ranging from diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Law, 2000). HUS is the leading cause of renal failure in children worldwide, and is often associated with renal or non-renal late sequela in patients who survive acute infections (Williams, Sreedhar, Mickell, & Chan, 2002). The infections usually develop around three days after consumption of contaminated food. Diarrhea may turn bloody after 1 to 2 days, and if HUS emerges, it follows HC in 5 to 7 days (Melton-Celsa, Mohawk, Teel, & O'Brien, 2011). The subset of STEC that is known to cause these severe clinical manifestations in human is referred to as Enterohemorrhagic *E. coli* (EHEC). *E. coli* O157 is the most recognized within the EHEC group due to its high association to HUS (Orth & Würzner, 2006). However, outbreak data reported by the Centers of Disease Control and Preventions (CDC) public health laboratory from 1983 to 2002,

supported six non-O157 strains that have emerged to cause increasing cases of HUS (Brooks et al., 2005). Non-O157 strains have been reported to cause severe illness, up to 60%, in several countries. These strains are O26, O111, O103, O121, O45 and O145, also known as the "Big 6" (Delannoy, Beutin, & Fach, 2013).

1.4.2 STEC virulence factors

The production of shiga-toxin (stx) is the most significant virulence characteristic for the development of HUS. Shiga-toxins target the kidney and disrupt microvascular endothelial homeostasis (Orth & Würzner, 2006). There are two types of shiga-toxins, stx1 and stx 2. Stx 1 is structurally similar to the shiga-toxin produced by *Shigella dysenteriae* (Orth & Würzner, 2006). There are at least 100 *E. coli* strains that present *stx* genes (Perelle, Dilasser, Grout, & Fach, 2007). These genes (*stx*1 and 2) are known to be horizontally transferred by lysogenic bacteriophage; therefore, it is suggested that any *E. coli* strains can acquire them (Paton & Paton, 1998). Although shiga-toxin production is essential, it is not sufficient in determining the pathogenicity of STEC. Another virulence factor is the myriad of adhesins that aid in the attachment of bacteria on the microvillus brush border of enterocytes (Melton-Celsa et al., 2011). One adhesin that is well known for its significant role in O157 pathogenesis, is *E.coli* Attachment Effacement (*eae*) gene that encodes for intimin (Orth & Würzner, 2006). There are more virulence factors related to STEC such as T3SS, other adhesins (Iha, Saa etc.), intimin receptors and immune modulators (NleC, NleE etc.) that play significant roles in the invasion and colonization of the host cell (Melton-Celsa et al., 2011). Some of these virulence factors (intimin, intimin receptor, T3SS and other effectors) are coded by genes located in a cluster called Locus of Enterocyte Effacement (LEE) Pathogenicity

Island (Melton-Celsa et al., 2011). It was suggested that the classification of EHEC within STEC is decided by the presence of LEE in their genome (Whittam, Wachsmuth, & Wilson, 1988).

1.4.3 STEC invasion, colonization and survival

Upon ingestion of contaminated food or from contact with contaminated live animals, the acid tolerance of STEC bacteria enables their survival in the stomach. A low infectious dose (less than 100 organisms) is enough to establish infection (Paton & Paton, 1998). As bacteria enter the intestine, they express stx and a variety of adhesin that induce cytokine production causing inflammation (Whittam et al., 1988). Intimin is the only adhesin that can produce A/E lesions, a necessary process for the colonization of animal guts, and increase the chance of intestinal damage (Melton-Celsa et al., 2011). Once the bacterium reaches the host cell intestinal epithelium, the T3SS delivers various effectors called *E. coli* secreted proteins (Esp), that alter host cell functions (Melton-Celsa et al., 2011). Some of these effectors such as Tir are intimin receptors and bind to intimin to cause cytoskeletal rearrangement of the host cell membrane to form a pedestal for the bacterium to invade the cell (Garmendia, Carlier, Egile, Didry, & Frankel, 2006). Other effectors interfere with host cell signaling and host cell transcription (Melton-Celsa et al., 2011). After secretion of Stx in the intestine, some of the toxins crosses the intestinal barrier through the enterocyte. The toxin is carried by the blood where it binds to receptors on the kidney and central nervous system (Brigotti et al., 2010). The toxin induces apoptosis of glomerular kidney cells, the primary cause of HUS and inflammation in the brain that disrupts normal function of neurons (Kaneko et al., 2001). Furthermore, lipopolysaccharide (LPS) of STEC induces proinflammatory cytokine responses which include upregulation of Gb3 (toxin receptor) and hypercoagulation accelerates the development of HUS (Zoja, Buelli, & Morigi, 2010).

1.4.4 Recent *E. coli* outbreaks

The STEC group is predominantly found in produce and beef products (Centers for Disease Control and Prevention, 2018a). The most infamous STEC outbreak happened in 1993 when STEC O157:H7 was found in Jack in the Box beef patties and infected over 700 people and led to four deaths of children (Centers for Disease Control and Prevention, 2001). However, leafy greens such as romaine lettuce and spinach are the biggest sources of STEC outbreaks, due to these products having direct contact with soil and water, and they are often consumed raw. Multiple studies suggested that *E. coli* O157:H7 are able to survive and invade plant tissues, specifically seedlings of lettuce (Franz et al., 2007; Jablasone, Warriner, & Griffiths, 2005; Johannessen et al., 2005; Solomon, Yaron, & Matthews, 2002). In fact, Solomon et al. (2002) reported that once *E. coli* are able to contaminates lettuce, it will be internalized through the root system of the plant into the edible leaf of the lettuce, making it impossible to remove through washing. A recent outbreak also shows that STEC O121 and O26 can survive in flour. Some cases from STEC result in deaths due to the fatal symptoms of HUS and kidney failure (Centers for Disease Control and Prevention, 2018a). These findings show that STECs are a significant public health threat associated with vegetable crops, especially due to their high mortality rate in children under five.

In Cambodia, limited research is done on STEC in food reservoirs. However, a study by Widmer et al. (2013) explored the prevalence of *E. coli* in surface water in Southeast Asia (Cambodia, Thailand, Vietnam and Indonesia). The findings revealed that 3.9% of the *E. coli* discovered were positive for one or more virulence genes (stx1, eaeA and elt) and were classified as STEC, EPEC or ETEC (Widmer et al., 2013). Moreover, a longitudinal study between 1958 and 1992 attempted to isolate pathogenic *E. coli* from diarrheal patients in Asia. The findings

demonstrated the presence of 3,065 strains of ETEC, EIEC, EPEC and STEC in the stool samples (Tamura, Sakazaki, Murase, & Kosako, 1996). These findings show that although pathogenic *E. coli,* such as STEC are rarely studied in food, they have been identified in water sources of Southeast Asia, and have caused diseases in the population.

1.4.5 STEC epidemiology and surveillance

Risk of STEC infection varies with the presence and the combination of virulence genes within the serotype. Although some serotype with *stx* and LEE-positive strains are associated with a higher risk of severe illness, other virulence gene combination (even those lacking *stx* gene) are also capable of causing severe illness (de Boer et al., 2015). Moreover, risk of infection is also affected by the host's characteristics. For instance, children below five have the highest risk of developing HUS, which might lead to kidney failure and death, from STEC infection (Nataro & Kaper, 1998).

As observed from the surveillance data of developed nations, certain serogroups of STEC are more common in causing illness than others. In the United States, 44% of foodborne illnesses are caused by EHEC O157 and 49.9% are caused by the "Big 6" (e.g. EHEC O26, O111, O103, O121, O45 and O145) (Centers for Disease Control and Prevention, 2015). In the EU, the EHEC serogroups O157, O26, O103, O113, O146, O91 and O145 are most frequently found in food outbreaks, with serogroup O157 representing half of the STEC related outbreak cases (European Centre for Disease Prevention and Control, 2016). These data do not currently exist in Cambodia since measures to detect STEC in the food chain are not in place. Consequently, the risk of STEC and their contamination rate within the food chain remains unknown. Therefore, a study to explore

the presence and concentration of STEC in the Cambodian food chain should be initiated in order to mitigate child mortality within the country.

1.5 Food safety in Cambodia

Approximately 20% of Cambodia's population is living in poverty (National Institute of Statistics Cambodia & Directorate General for Health, 2015). One of the main causes of poverty is the loss in productivity of citizens caused by poor health, especially those living in rural areas. Diarrheal diseases are major contributors to most health issues faced by the rural populations in the country. Furthermore, an estimated 10,000 deaths, or more, in children under five years are caused by diarrheal diseases in Cambodia every year (United Nations Children's Fund, 2018). Yet, no current studies have fully characterized the attribution and causal pathways for these deaths. Traditionally, diarrheal disease has been linked to unsafe water that is consumed as drinking water, used for washing produce and/or the irrigation of crops, as well as lack of basic knowledge on hygiene and sanitation (Food and Agriculture Organization of the United Nations, 2015). However, up to 40% of diarrheal cases in developing countries are not caused by either poor quality water or inadequate sanitation, but are due to poor safety and quality of food (Grace, 2015). The main agents of food contamination are bacterial pathogens (Havelaar et al., 2015). The leading pathogens that cause the highest number of foodborne illness cases around the globe are norovirus, *Campylobacter* spp., ETEC (Enterotoxigenic *Escherichia coli*) and non-typhoidal *Salmonella enterica* (Havelaar et al., 2015).

Although the WHO Global Burden of Foodborne Disease report delivered helpful information on foodborne disease trends and prevalence of food hazards, there has been minimal effort on capturing these data at a country-specific level in Cambodia. Furthermore, the WHO

report classified Cambodia into the WPR B region—a region consisting of multiple developed nations, such as China and the Republic of Korea, that most likely affected the mortality rate averages—which is not fully representative of Cambodia's overall burden or situation.

Very limited studies on foodborne hazards can be found in Cambodia, especially those concerning biological hazards. Capturing data on foodborne diseases in Cambodia is highly challenging due to the lack of surveillance systems on foodborne diseases and outbreaks. The 2018 Salmonellosis Global Status report, which outlined data associated with cases and outbreaks of salmonellosis per country, is one of the only published documents that reported foodborne disease levels in Cambodia. According to this report, only five cases of salmonellosis in Cambodia have occurred since 1981 (Berger, 2018). This is strong evidence that foodborne diseases are being underreported and neglected in Cambodia. Furthermore, there is also a strong evidence that these pathogens have have caused high number infections in Cambodian patients. A 2007 study by a collaboration of hospitals in Phnom Penh, Cambodia, revealed that *Escherichia coli* and *Salmonella enterica* are amongst the most common bacterial pathogens detected in patients with bacterial infections (Vlieghe, 2014). From the mentioned reports and studies, it is supported that foodborne disease is an occurring public health issue in Cambodia.

1.5.1 Food safety policy in Cambodia

Food safety in Cambodia is under the jurisdiction of the Ministry of Health (MoH), Ministry of Agriculture Forestry and Fisheries (MAFF), Ministry of Industry, Mines and Energy (MIME), and Ministry of Commerce (MOC). Furthermore, Cambodia adopted the Codex Alimentarius framework for standards on food safety. In fact, a Cambodia National Codex Committee (CNCC) was established within the MOC to ensure coordination of food safety matters within these industries (Ministry of Agriculture Forestry and Fisheries, 2004). In 1993, the

Ministry of Commerce and Good Manufacturing Practice (GMP) developed several frameworks for food safety within the food production system (e.g., management and control of food safety and quality) (Ministry of Agriculture Forestry and Fisheries, 2004). There are also regulations regarding labeling of food products and third-party certification system set in place prior to 1993 (Ministry of Agriculture Forestry and Fisheries, 2004). These regulations, though they exist, are poorly enforced due to several reasons: (1) most personnel that engage in food safety monitoring do not have the training or minimum qualification in food safety; (2) the lack of a surveillance systems to monitor foodborne contamination and diseases occurring in the country (Ministry of Agriculture Forestry and Fisheries, 2004); and (3) the lack of infrastructure in food selling facilities and education on basic hygiene practices. Each of these factors enhance the food safety risk that might contribute to the development of foodborne disease within the population. First, the lack of food safety training of monitoring personnel would decrease the potential of detecting food safety threats. Moreover, the lack of physical and organizational infrastructure in food safety facilities would also support the contamination of food products, mainly from cross-contamination. Lastly, the lack of local surveillance system would hinder the ability of regulatory bodies to record and establish control on occurring foodborne outbreaks.

Cambodia is currently drafting their national food safety law, which was prepared in partnership with the United Nation's Food Agricultural Organization (FAO). It was proposed in 2015 and has been going under revision for three years but is yet to be approved (Vo, 2018). The factors mentioned emphasize how regulations and politics can greatly impact the public health status of the country. Unless immediate regulatory interventions are identified, prioritized and implemented, food safety issues will continue to persist within Cambodia's food value-chain.

1.5.2 Vegetable value-chain in Cambodia

Cambodia produces around 6.18 ton/hectare of vegetables annually and a majority of this production is consumed locally (Underhill, 2013). The Cambodian diet varies with socio-economic status, but most of the population consumes more vegetables and fish than animal source foods. This is commonly due to the lack of affordability of animal sourced products within the country. An estimate of 70% of vegetables sold in the markets are imported from export market of neighboring countries (i.e. Vietnam and Thailand) due to local farmers not being able to meet with the demands of the country (Sokhen, Kanika, & Moustier, 2004). The vegetable value-chain in Cambodia involves mainly farmers, collectors, distributors and vendors (Sokhen et al., 2004). More than 80% of the value-chain exchanges occur in the informal markets. Informal markets are markets that "escape effective health and safety regulation, are often untaxed and unlicensed" (Roesel & Grace, 2014). Informal markets can be found in all provinces of Cambodia and most vendors depend on the exchanges made in these markets as their only source of income. These markets are popular among the average Cambodian consumers because they provides] cheaper and fresher products compared to those sold in supermarkets. These markets also enable consumers to support local, trusted vendors that are often outcompeted by large supermarket retailers in the city (Roesel & Grace, 2014).

The vegetable value-chain in Cambodia begins with the farmers and ends with consumers (e.g. household, restaurant, hotels etc.) (Figure 1.3). Around 14% of the farmers grow their own vegetables and sell them to collectors or distributors (Sokhen et al., 2004). The rest of the farmers act both as growers and as collectors, meaning they sell both their personal vegetables as well as vegetables from other farmers. Collectors act as middlemen between farmers, distributors and vendors. Collectors transport vegetable crops using vehicles. There are two types of collectors: (1)

those that purchase vegetables from farmers in villages, as well as imported vegetables from neighboring countries (i.e. Vietnam and Thailand), and transport them to sell to distributors and vendors. These collectors also transport crops from local distributors to vendors; and (2) those that purchase from other collectors in the markets to sell to other collectors in the city (i.e. Phnom Penh, the capital city). Distributors purchase vegetables from collectors or farmers and sell them in bulks to the vendors. The vendors are stationed in informal markets, where most Cambodian consumers purchase their regular servings of vegetables from. Food businesses such as restaurants and hotels may purchase from distributors as the number of vegetables needed is higher than the average household consumers (Sokhen et al., 2004).

1.5.3 Food safety in horticulture

Vegetables and other horticulture products are known to be common sources of foodborne pathogens, due to the fact that they are often consumed unprocessed or raw (Olaimat & Holley, 2012). Recent data suggest that there is an increasing trend of outbreaks related to fresh produce worldwide, mostly due to *Salmonella enterica* and *E.coli* O157:H7 (Franz & van Bruggen, 2008). In developed nations, where control of food is stringent, foodborne outbreaks from produce remains prevalent. In the United States, produce caused the highest numbers of foodborne disease outbreak from 1990 to 2004, exceeding meats and seafood (Olaimat & Holley, 2012). In Europe, produce caused several international multi-countriy outbreaks between 1992 and 2000 (Franz & van Bruggen, 2008). Similar evidence is available in developing countries in Southeast Asia; the consumption of fresh produce is increasingly attributed to diarrhea in Vietnam (Roesel & Grace, 2014). The average Cambodian diet, which is mostly composed of raw vegetables, suggests that fresh produce is likely to contain biological hazards and that controls are necessary to mitigate future illnesses and outbreaks.

Further, the prominent use of manure as organic fertilizer for vegetables grown in Cambodia enhances the challenges in food safety at the farm level. According to recent statistics by The World Bank, more than 80% of farmers use manure from animals as vegetable fertilizer in Cambodia (Eliste & Zorya, 2015). Manure is a cost-effective way for smallholder farmers to utilize the byproduct of their livestock to supply nutrients for the crops. However, manure is a source of microbial pathogens (Franz & van Bruggen, 2008). STEC and *Salmonella enterica* are some of the main pathogens present in animal waste that can contaminate vegetables after application of manure. These pathogens can persist in the vegetables if manure is inadequately composted or not applied properly during cultivation (Franz & van Bruggen, 2008; Natvig, Ingham, Ingham, Cooperband, & Roper, 2002). The absence of regulation and education on handling manure for application on vegetables for farmers and vendors along the vegetable value-chain puts the country at risk for diseases and other health conditions.

Following harvest, food safety remains a problem as most vegetables are exposed to unsanitary practices. There are no recommendations on how to safely transport vegetables between provinces, so vegetables are often transported as open cargo, exposed to dirt and dust, and other disease carriers (e.g. live pests, bird droppings). Moreover, most vegetable crops purchased and consumed in Cambodian households are purchased through informal markets (United States Agency for International Development, 2015). In these markets, food safety is often absent and not practiced by the people involved, due to lack of educational and infrastructural capacity. These markets lack the infrastructure necessary to provide safe and clean food, for instance, the walls and floors of the markets are dirty and full of cracks, which provides niches for microorganism growth. Some vendors do not have tables to display their vegetable crops on, resulting in most vendors selling on the ground. Furthermore, these markets do not have pest controls and are

infested with insects and rodents. Stray cats and dogs also freely roam the streets where these markets are located. These animals might be potential carriers of critical foodborne pathogens that might contaminate vegetable in the markets.

The food safety concern in the informal markets are intensified by the lack of education of vendors about hygiene practices. Practices such as selling raw meat directly beside fresh vegetables, handling vegetables and meats without gloves and not washing bins after use are common practices in these markets. These practices might introduce cross-contamination of biological hazards, particularly bacteria onto the vegetables. Cross-contamination during food preparation and handling has commonly been a major cause of outbreaks in fresh produce in the United States and United Kingdom (Evans, Ribeiro, & Salmon, 2003). Findings from existing literature revealed that meat products sold in the informal markets have historically been contaminated with pathogens such as *Escherichia coli*, *Salmonella enterica* and tapeworm cysts (Roesel & Grace, 2014). Moreover, a study done on raw poultry in Cambodian informal markets revealed that 88.2% of samples were positive for *Salmonella enterica* and 80.9% of samples were positive for *Campylobacter* spp. Quantification data from this study also showed concentrations of 3-4 $\log_{10}$ CFU/g for *Salmonella enterica* and 7-8 $\log_{10}$ CFU/g for *Campylobacter* spp. (Lay, Vuthy, Song, Phol, & Sarthou, 2011). These findings emphasize the high possibility of cross-contamination in the market. Improper handling of raw meat or poultry might potentially introduce cross-contamination of the same pathogen to vegetables that are sold within the same area. Once a few vegetables are contaminated, it is likely for cross-contamination to further occur to affect the whole lot.

Informal markets are an integral part of the Cambodian lifestyle, culture, and economy. It also serves as the final point in the value-chain before vegetable crops enter households. Therefore,

interventions introduced at markets, even as minimal as providing basic sanitation supplies or training on safe food handling, is critical and will likely improve public health outcomes for the country. Furthermore, investigation on bacteriological hazards found in vegetables sold in these markets are a starting point in calculating the risk present within the Cambodian vegetable value-chain.

1.5.4 Food safety research in vegetable crops in Southeast Asia

Research concerning food safety in Cambodian vegetable crops has mostly focused on pesticide use (Jensen, Konradsen, Jørs, Petersen, & Dalsgaard, 2011; Thetkathuek, Suybros, Daniell, Meepradit, & Jaidee, 2014), heavy metals in drinking water (S. Khan, Cao, Zheng, Huang, & Zhu, 2008), and parasitic infections (Schär et al., 2014). However, there are limited studies that focus on biological hazards. The 2018 Salmonellosis Global Status report only recorded five cases of Salmonellosis in Cambodia since 1981 (Berger, 2018). This suggests that bacterial pathogens are being underreported and underexplored in all aspects of the country, especially in informal settings such as the informal markets.

Only a few studies discuss the bacteriological hazards present in vegetables sold in Cambodian informal markets. Set et al. (2012) assessed the load of *E. coli* and *Salmonella enterica* in cabbage collected from farms, distributors and vendors in Phnom Penh (Table 1.1). Findings showed that the cabbage collected has 2.98 $\log_{10}$ CFU/g and 3.00 $\log_{10}$ CFU/g of *E. coli* and *Salmonella* enterica, respectively (Table 1.2, Table 1.3). Chrun, Hosotani, Kawasaki, and Inatsu (2017) investigated the contamination of coliforms (e.g. *Escherichia coli*, *Cronobacter sakazakii*, and *Enterobacter* spp.*),* opportunistic non-*Enterobacteriaceae* spp., *Enterococcus* spp., *Staphylococcus* spp. and *Listeria* spp. in fermented vegetables. Results revealed that the highest contamination was by *Enterococcus spp*., followed by *Bacillus spp.* and *E. coli* with 31%, 24%

and 10%, respectively (Chrun et al., 2017). However, it is important to highlight that these studies are limited in scope: (1) The study by Set et al. (2012) was only targeting a single type of vegetable (e.g. cabbage) and was conducted on an extremely limited number of samples, therefore was not a good representation of the vegetable crops in the informal markets, (2) The study by (Chrun et al., 2017) was conducted on fermented vegetables, a food matrix that are not comparable to fresh vegetables (due to its acidity, presence of competitive microorganism etc.), and (3) Both studies presented methodological differences which impaired the comparison between results. A market-based study in Cambodia, focusing on bacteriological hazards on various types of vegetables has not been scientifically explored.

Other developing nations in Southeast Asia, such as Vietnam, the Philippines, Thailand, and Malaysia have conducted informal market-based studies to evaluate bacterial contamination in a wide range of vegetable crops (Table 1.1). A study in Vietnam explored the concentration of aerobic bacteria and *E. coli,* as well as the prevalence of *Salmonella enterica.* and several parasites in twelve different vegetables collected from three different informal markets (Chau et al., 2014). Findings revealed *E. coli* concentrations up 6 $\log_{10}$ CFU/g in all vegetables sampled, and 17.5% of total samples tested positive for *Salmonella enterica* (Table 1.2, Table 1.3). P. G. Vital, K. G. Dimasuay, K. W. Widmer, and W. L. Rivera (2014) investigated the concentration of *E. coli* and *Salmonella enterica* in five types of fresh vegetables collected from five informal markets in the Philippines. This study found *E.coli* concentrations up to 4 $\log_{10}$ CFU/g in 16.4% positive samples, and 24.4% of *Salmonella enterica.* positive samples (Table 1.2, Table 1.3). Moreover, a similar study conducted in Thailand (Ananchaipattana et al., 2012) that showed 14% positive prevalence rate for *E. coli* and 6% positive prevalence rate for *Salmonella enterica*, respectively (Table 1.3).

Although the studies from Vietnam, the Philippines and Thailand were extensive, none of them have analyzed *Salmonella enterica* to a serovar level. A study by Salleh et al. (2003) in Malaysia was the only informal market-based study conducted on vegetables that serotyped *Salmonella enterica*. Results suggested that out of 35.7% *Salmonella enterica* positive samples, the most common serovars recovered were *S. weltevreden*, *S. agona* and *S. senftenberg* (Table 1.3). This result is unexpected as these serovars are not commonly discovered in clinical samples in the United States and the European Union (European Centre for Disease Prevention and Control, 2014; National Center for Emerging and Zoonotic Infectious Diseases, 2013).

Although Southeast Asian countries (i.e. Cambodia, Vietnam, the Philippines, Malaysia and Thailand) are culturally similar, several factors contribute to the fact that regional data cannot be extrapolated to fit the context of Cambodia. First, there is a vast difference between the socioeconomic status between countries (i.e. economy and infrastructure). Many Southeast Asian countries are now considered middle-income countries with developing physical and organizational infrastructure. Conversely, Cambodia is transitioning from a low-income to a middle-income country, with less infrastructure in place. Further, methodological differences exist between each study which results in data not being comparable between countries. These differences emphasize that the data from studies conducted in other countries would not be an accurate representation of the bacterial contamination in Cambodian informal markets (Table 1.1). Current food safety research conducted in Cambodia, has not fully addressed the gaps discussed previously. For these reasons, a country-specific study that focuses on the presence and concentration of bacterial hazards in fresh vegetables sold in Cambodian informal markets is needed. Moreover, a complementary survey study that investigates the hygiene practices of Cambodian vegetable value-chain actors would also be beneficial to identify potential

contamination points within the chain. This data will be beneficial in understanding if biological hazards observed at the informal chain might be a result of the lack of food safety practices within the value-chain. Consequently, both microbial and survey data will be valuable resources in the development of future intervention strategies to mitigate the likelihood of bacterial contamination in the Cambodian vegetable value-chain. The results will also serve as a platform in the development of epidemiological surveillance within the country of Cambodia.

Table 1.1 A review of informal market-based studies that have been conducted on fresh vegetables in Southeast Asia.

Country	Vegetable types	Methods	Reference
Cambodia	Chinese cabbage (*n*= 9)	*E. coli* enumeration: rinsed using Mossel-Bouillon broth and plated on Eosin Methylene Blue (EMB) agar. *Salmonella enterica* enumeration: rinse in buffered peptone water (BPW) and plated on DHL, Bismuth sulfide (BS) and Brilliant Green (BG) agars. Confirmation by biochemical tests.	(Set et al., 2012)
Vietnam	Mustard greens, celery, amaranth, cilantro, water spinach, rice paddy, cilantro, basil, centella, lettuce, watercress, iceberg lettuce (*n*=108)	*E. coli* enumeration: rinsed using 0.1% peptone water and plated on EMB agar. Confirmation using biochemical tests. *Salmonella enterica* detection: PCR targeting *invA* gene.	(Chau et al., 2014)
The Philippines	Bell pepper, cabbage, carrot, lettuce and tomato (*n*= 300)	*E. coli* detection and enumeration: rinsed using 0.1% BPW and plated on MacConkey (MAC) agar. *Salmonella enterica* detection and enumeration: MPN methods using Rapapport-Vassiliadis (RV) broth	(P. G. Vital, K. G. B. Dimasuay, K. W. Widmer, & W. L. Rivera, 2014)
Malaysia	Vietnamese coriander, centella, chinese celery and water spinach (*n*=112)	*Salmonella enterica* detection: rinsed using BPW, selective enrichment in RV and mannitol selenite cystine broth. Streaked on BS, Rambach, Hektoen enteric and Xylose Lysine Deoxycholate (XLD) agars. Presumptive positive isolates were serotyped.	(Salleh et al., 2003)
Thailand	Leafy greens (*n*=38)	*E. coli* detection: Selective enrichment in modified *E. coli* broth and streaked on Rainbow O157, Chrom O26-O157, XM-EHEC and MacConkey Agar with Sorbitol, Cefixime and Tellurite (CT-SMAC) agars. Confirmation using biochemical tests. *Salmonella enterica* detection: pre-enrichment in Enterobacteriaceae Enrichment Mannitol broth, followed by selective enrichment in Tetrathionate and RV broth. Streaked on Deoxycholate Hydrogen Sulfide Lactose, BS and BG agars. Confirmation using API 20E.	(Ananchaipattana et al., 2012)

Table 1.2 A review of prevalence and concentration of *E. coli* in fresh vegetables sold in informal markets in Southeast Asia

Country	**Contamination level (log_{10} CFU/g)**	**Percentage of *E. coli* positives (%)**	**Reference**
Cambodia	2.98	NA[a]	(Set et al., 2012)
Vietnam	6.30	NA[a]	(Chau et al., 2014)
The Philippines	1.26 - 3.63	16.40	(Pierangeli G Vital et al., 2014)
Thailand	NA[a]	44.00	(Ananchaipattana et al., 2012)

[a]Value is not available (NA) in current publications and studies

Table 1.3 A review of prevalence and concentration of *Salmonella enterica* in fresh vegetables sold in informal markets in Southeast Asian countries

Country	**Contamination level**	**Percentage of *Salmonella enterica* positives (%)**	**Reference**
Cambodia	3.00 log CFU/g	NA[a]	(Set et al., 2012)
Vietnam	NA[a]	17.59	(Chau et al., 2014)
The Philippines	2.34 log MPN/g	24.40	(Pierangeli G Vital et al., 2014)
Malaysia	NA[a]	35.71	(Salleh et al., 2003)
Thailand	NA[a]	6.00	(Ananchaipattana et al., 2012)

[a]Value is not available (NA) in current publications and studies

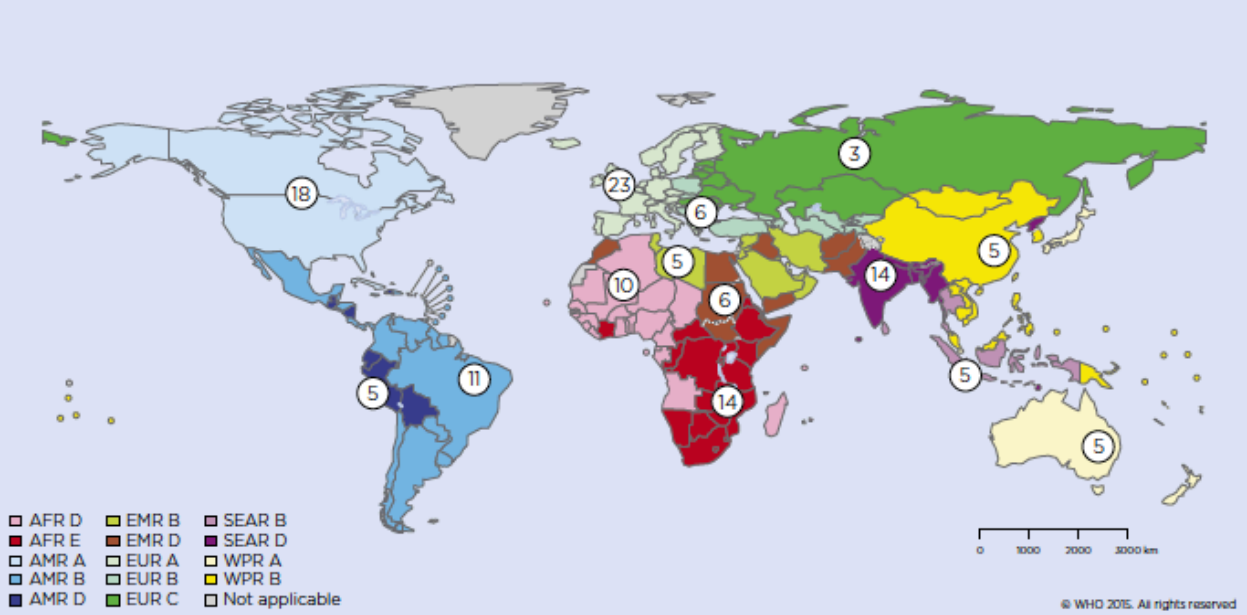

Figure 1.1. Sub-regions as classified by the WHO for foodborne disease burden estimation

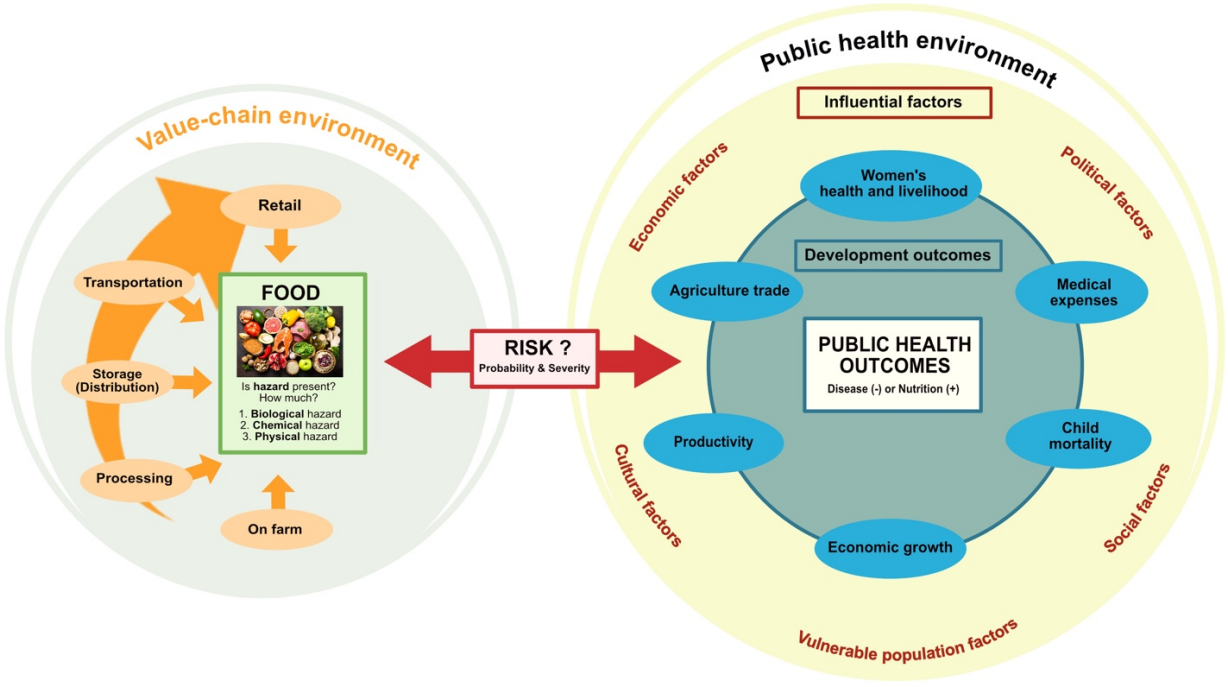

Figure 1.2. Food safety hazards in relation to risk and public health outcomes

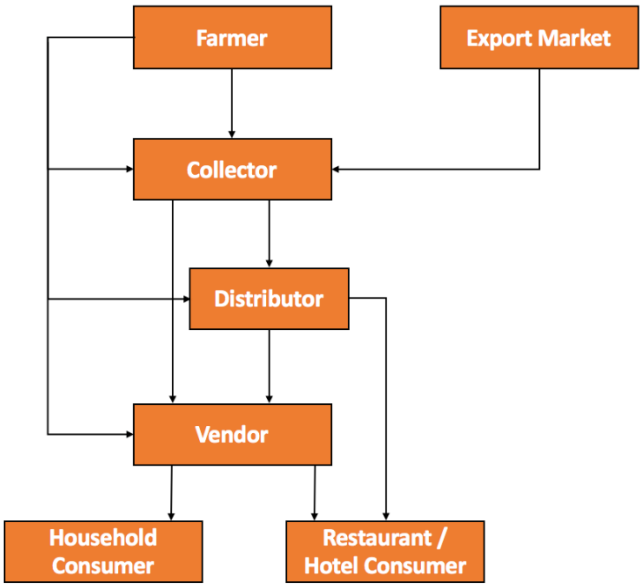

Figure 1.3. Vegetable value-chain in Cambodia as described by Sokhen et al. (2004)

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Chapter 2 - Defining the flow and food safety behaviors of actors in the Cambodian vegetable value-chain

2.1 Introduction

Food safety, as a discipline, is a multi-stakeholder activity. As a food product moves through a specific agriculture value-chain (i.e. on farm, processing, storage, transportation and retail), it is critical that all agriculture value-chain actors actively contribute to ensuring the safe handling of all food products. Food safety is effectively maintained from "farm to fork" by the implementation of multiple pre-requisite programs and/or interventions and practices, such as sanitation programs, good agriculture practices/good manufacturing practices (GAP/GMP), temperature control, sanitizers, and heat treatments (Orriss & Whitehead, 2000; Raspor, 2008). These types of multi-hurdle approaches, when implemented, are more effective at reducing and eliminating food safety hazards than individual hurdles (Leistner, 1995). Consequently, the multi-hurdle approach provides a safer product and a shared contribution by different value-chain actors; however, these types of approaches require proper investments in food safety capacity within agriculture value-chains. Unfortunately, many developing nations around the world significantly lack the necessary investments to create food safety capacity, and therefore face large obstacles in terms of the control of foodborne hazards in multiple food value-chains.

In the country of Cambodia with a population of nearly 15.4 million people, child mortality rates are high, with over 10,000 deaths per year being attributed to diarrheal diseases (Food and Agriculture Organization of the United Nations, 2015; United Nations Children's Fund, 2018). A majority of the diarrheal disease cases in the country have not been traced back to a particular source; however, there is a high likelihood that contaminated foods contribute heavily to the occurrence of diarrheal diseases in developing nations (Grace, 2015). The Cambodian diet

predominantly consists of fresh, uncooked vegetables which are commonly purchased through informal market systems. Informal markets are markets that "escape effective health and safety regulation, and are often untaxed and unlicensed" (Roesel & Grace, 2014; Sokhen et al., 2004). In fact, a majority of vegetables in Cambodia are sold through these markets (Sokhen et al., 2004). Informal markets commonly source vegetables through the countries' vegetable value-chain. This value-chain consists of several actors, all of which have a role to play in food safety. These actors include farmers, collectors, distributors and vendors (Sokhen et al., 2004). Farmers grow and harvest vegetables, which are then transported by the collectors to the distributors that sell vegetables in bulk. Collectors also procure vegetables from the neighboring countries of Thailand and Vietnam. Finally, vegetables are transported from the distributors to the markets by the vendors or by other collectors before they reach households or mass consumers (i.e. hotels or restaurants) (Figure 2.1).

Contaminated vegetables have a high potential to cause foodborne diseases and have been attributed to multiple outbreaks worldwide (Franz & van Bruggen, 2008; Olaimat & Holley, 2012). Without proper handling along the value-chain, pathogens can be easily transferred from the natural environment to vegetables, increasing the consumer's likelihood of acquiring a foodborne illness. Further, the control of the vegetable value-chain in Cambodia is deficient in areas of policy, guidelines, and education (Ministry of Agriculture Forestry and Fisheries, 2004), and recommended hygiene and sanitation practices are frequently neglected. Consequently, stakeholders within the vegetable supply-chain are unaware of the food safety risks and may not use proper handling practices to minimize cross-contamination risks. For these reasons, it was necessary to conduct an explorative review of the Cambodian vegetable value-chain that thoroughly defines the flow of the products within the value-chain and provides insights on

potential contamination points. For the purpose of this study, comparisons of practices between actors were not evaluated.

The two main objectives for this study include: (1) to specify and describe the flow and the behaviors of stakeholders within the Cambodian vegetable value-chain by personal interviews; (2) to identify practices that can potentially contribute to the cross-contamination of vegetables moving through the value-chain. Information from this study will be a valuable resource for the creation of food safety recommendations that will aid in the prevention of potential foodborne illness attributed to vegetable products in Cambodia.

2.2 Materials and methods

The research protocol and data collection tools were reviewed and approved by the Institutional Review Board at Kansas State University (IRB #8821.1).

2.2.1 Participants and data collection

A survey tool with four different questionnaires was designed to investigate food safety practices of Cambodian farmers, collectors, distributors and vendors. Each questionnaire consists of different sets of questions focusing on sanitation and hygiene practices at the various steps of the vegetable value-chain. The questionnaires were created in an online software (KoBoToolbox), "a suite of tools for field data collection" developed by the Harvard Humanitarian Initiative (KoBoToolbox, 2019). Despite the presence of imported vegetables, the survey is focused on the practices conducted by local stakeholders. For this reason, the handling of imported vegetables was not evaluated at the production level, but was considered for local collectors, distributors, and vendors. Additionally, an observational assessment was conducted in the informal markets to examine the food safety practices of vendors, as well as hygiene condition of bathroom facilities.

Survey questionnaires were developed in English and translated into Khmer (the local language) and orally administered—by university students from the Royal University of Agriculture (RUA), Phnom Penh, Cambodia—to participants in a personal one-on-one interview. The survey was administered orally to gain more reflective answers, ensure the understanding of respondents on complex questions and to have more control over response rate. Students were trained on the topic of the questionnaires prior to survey administration. Participants were classified as one of four vegetable value-chain actor types, initially identified by Sokhen et al. (2004): (1) farmers, (2) collectors, (3) distributors, or (4) vendors. Informed consent as well as participant's category was ensured by verbal confirmation before survey administration. Furthermore, the subjects were provided with a small monetary gift for their participation, which was not made known to them until the end of the study to reduce bias in responses. At the end of the data collection, a total of n=102 farmers, n=21 collectors, n=30 distributors, and n=100 vendors were interviewed. Interviews were conducted in Battambang (rural; n=137) and Siem Reap (peri-urban; n=116) provinces. Differences in interview numbers were based upon the number of market vendors in each location. Further, visual assessments were conducted on n=52 (n= 15 in Battambang and n=37 in Siem Reap) vegetable vendors.

Battambang is located in the Northwest region of Cambodia (Belfield, Martin, & Scott, 2013) and is considered a rural province in Cambodia. Conversely, Siem Reap, which is also located in the Northwest region of the country, is a peri-urban province (Mao, Grunfeld, DeLacy, & Chandler, 2014). The rationale for selection of these provinces was based upon (1) their classification as United States Agency for International Development (USAID) Feed the Future Zones of Inquiry; (2) differences in socioeconomic status and demographics (National Institute of

Statistics Cambodia & Directorate General for Health, 2015); and (3) a parallel study of the microbial contamination of vegetables was also being conducted in these two provinces.

2.2.2 Farmer questionnaire

The farmer questionnaire examined the food safety practices of farmers with a focus on the cultivation and harvesting of vegetable crops. Questions included the type of vegetables grown, type of soil amendments used, cleaning and storage practices for harvested vegetables, etc. (select questions are outlined in Table 2.1). Conditional questions were included to capture data on organic fertilizer use, such as aging time (for compost) and time interval between application and harvest (for fresh manure).

2.2.3 Collector questionnaire

As collectors are generally the value-chain actors most heavily involved with the transportation of crops, the collector questionnaire focused on the food safety practices during the transport of vegetables. Questions included the following: the person and location the vegetables were purchased from; the cleaning, inspecting and sorting process of vegetables received, the type and cleaning practices used for transport vehicles; the storage method for received vegetables, etc.

2.2.4 Distributor and Vendor questionnaire

Both the distributor and vendor survey questionnaires focused on the handling of vegetables during selling. Questions included the person and location the vegetables were purchased from, the cleaning, inspecting and sorting process of received vegetables, the basic food safety and hygiene practices during preparation of selling areas, etc. Visual assessments for vendors consisted of observations on basic food safety practices (e.g. handwashing, tarp condition) and stall sanitation (e.g. vendors location within the market, etc.). Visual assessment questions on bathrooms consisted of observations on facility condition and availability of handwashing stations.

Farmers and collectors in the study were randomly selected from multiple villages in Battambang (BB) and Siem Reap (SR). Distributors were selected from a distribution center in BB, which is the main distributor center for the Northern region of Cambodia. Vendors were mainly selected from six markets in BB and SR, where microbial analysis samples for a separate study were collected. Finally, gender and province data were also collected in each questionnaire, and data were disaggregated accordingly.

2.3 Results

2.3.1 Overall results

Farmers

Farmers that participated in the study (n=102) consisted of n=30 males and n=72 females. Moreover, n=50 were situated in BB, and n=52 were situated in SR. Findings suggest that leafy greens and salad vegetables (e.g. lettuce, cucumber and Chinese broccoli; 74%), podded vegetables (e.g. long beans; 20%) and flower buds (e.g. cauliflower; 4%) are the major vegetable crops cultivated by the farmers in this study (Figure 2.3). A majority of farmers interviewed utilized inorganic fertilizer, followed by fresh manure, and "composted" animal waste (Table 2.1, Figure 2.4). Findings suggest that the median time interval between application of fresh manure and harvest was 3 days, with a minimum value of 1 and a maximum value of 365 days. Moreover, the median aging time of "compost" was 13 days, with a minimum value of 3 day and a maximum value of 120 days. Upon further conversation with the farmers, it was implied that no specific composting procedure was routinely followed. The information about the composting process, however, was not observed or investigated in detail in this survey.

Responses about cleaning procedures revealed that more than half of the farmers (66%) do not clean harvested vegetables due to multiple reasons (Table 2.1, Figure 2.5). Farmers who did

indicate they clean vegetables primarily washed them with ground water (98%). In terms of storage, a high percentage (98%) of farmers do not store harvested vegetable crops on the farm for more than one day. Instead, most farmers sell or transfer ownership of harvested vegetable-crops multiple times a week (on harvest days) to different collectors (87%).

Collectors

Collectors are also defined as 'middlemen' for farmers, distributors, and vendors in the vegetable value-chain. It is important to note that fewer collectors exist in each province, due to the fact that one collector will work with several farmers. Collectors who participated in the study (n= 21) consisted of n=13 males and n=8 females. Moreover, n=11 were situated in BB and n=10 were situated in SR. Findings suggested that 77% of collectors solely obtained vegetables from farmers in Cambodia, 9% solely obtained vegetables from export markets (e.g. Thailand and Vietnam), whereas 14% sought from both. Moreover, a high percentage of collectors maintain a close business relationship with local farmers, as most of them (89%) purchased from the same farmers every time.

Responses on cleaning procedure questions showed that 71% of the collectors do not wash received vegetables (Table 2.1, Figure 2.5). The collectors who do wash vegetables do so by using either ground water (50%) or surface water (50%). After washing, a high percentage of collectors inspect vegetables for bruises or cuts (95%), and bruised vegetables are handled in several manners (Table 2.1, Figure 2.6). This includes cutting bruised vegetables, discarding and returning bruised vegetables to the sellers. In terms of storage, packing and transport of vegetables, 90% of collectors answered that they sort vegetables by type and pack them in separate containers. Further, approximately 81% of collectors do not store vegetables for more than one day. Vegetables are mostly delivered to distribution centers (67%) and informal markets (29%) using cars and trucks

(Table 2.1). Findings regarding sanitation of transport vehicles revealed that only 67% of the collectors acknowledged that they cleaned their vehicles prior to transportation.

Distributors

Distributors are the wholesalers who sell horticulture crops in bulk quantity. This is the first location where packed vegetables are being displayed in a retail setting for purchase to potential buyers (e.g. individual vendors or other collectors). There are only a few distribution centers in each region of Cambodia, and each distribution center will supply multiple markets in that region. The distributors in the study consisted of n=21 females and n=9 males, all of whom sell their vegetables at Pum Puy market in BB, a distribution center in the northern region of Cambodia (n=30). Distributors sourced vegetables from several suppliers: collectors (50%), export market (34%) and local farmers (16%) who delivered vegetables to the distribution centers. Vegetables mostly come to the distributor center in cars or trucks (Table 2.1). These findings show that distributors are sourcing vegetables from multiple value-chain actors, and that they are able to source from farmers or the export markets without the help of collectors. However, a close relationship between distributors and collectors was also observed, as most bought from the same collectors every time.

Cleaning procedure questions revealed that 87% of distributors do not wash received vegetables due to several reasons (Table 2.1, Figure 2.5). Distributors that clean vegetables use ground water (67%), surface water (33%) and municipal water (33%). Furthermore, approximately 97% of distributors also inspect crops for bruises. Bruised vegetables are most often cut by distributors before selling (Table 2.1, Figure 2.6). In terms of packing of vegetable crops, distributors also sort loose vegetables (i.e. vegetables that did not come packed from collectors) by type and pack them into different containers; indicating that distributors are mixing vegetables

from multiple sources. Packed vegetables are sold in bulk to buyers (i.e. collectors, vendors) in individual stalls in the distribution centers.

Findings from this survey revealed that most distributors are using multiple approaches for maintaining and cleaning stalls (Table 2.1). For example, most distributors replaced tarps used to display vegetables when there are signs of damage (57%). Additionally, 76% of distributors do not use gloves while handling vegetables, while the remainder use reusable fabric gloves. Leftover vegetables are mostly stored at room temperature (71%), in a container with ice (24%) or refrigerator (5%) to be sold the following day. Vegetables from the distribution center are sold to other collectors (70%) and vendors (50%).

Vendors

The vendors that participated in the study (n= 100) consisted of n=100 females, of which n=46 are situated in BB and n=54 are situated in SR. In the Cambodian vegetable value-chain, most vendors are stationed in informal markets as individual vendors. These informal markets can be found in multiple numbers in each province. Findings indicated that a majority of vendors obtain vegetables from collectors (71%), in comparison to distributors (23%). Additionally, findings revealed that some informal market vendors are also farmers, and therefore, are selling their own harvested crops. Vegetables are primarily transported to the informal market by motorcycle (Table 2.1). Approximately 68% of vendors admitted to not washing vehicles before transporting vegetables. Further, findings revealed that a high percentage of vendors do not wash vegetables after receiving (72%) (Table 2.1, Figure 2.5). Vendors that clean vegetables use ground water (46%), municipal water (33%) and surface water (21%) to wash vegetables. A high percentage of vendors also inspect received vegetables for bruises and cuts (74%). Further findings revealed that

most bruised vegetables are discarded by vendors, but there is also a fraction of bruised vegetables that are cut or sold as they are (Table 2.1, Figure 2.6).

The vendor survey also covered food safety practices during the selling of vegetables. Most vendors conduct cleaning activities in their stall before selling (Table 1). Moreover, more than half of the vendors (67%) indicated they replace tarps whenever damage occurred. Similar to distributors, 60% of vendors do not use gloves, while the remainder used reusable fabric gloves. After selling, 95% of vendors store the leftover crops in a container with ice (79%), in a container at room temperature (19%), or in a refrigerator (1%) to be sold the following day.

A visual assessment was also conducted in parallel with survey collections for *n*= 52 vendors (n=15 in BB, n=37 in SR) (Table 2.2, Figure 2.7). Visual assessments did not fully confirm vendor survey responses, as a majority of tarps being used in both markets had signs of damage. Moreover, visual assessments showed that a high percentage of vendors were observed conducting poor food safety practices. This included not removing their gloves and washing their hands after touching non-vegetable objects. Furthermore, observations of stall vicinity identified the presence of pests, a lack of handwashing stations—with 89% of handwashing stations consisted of a bucket of water that was being used to wash other objects as well (e.g. water was cloudy and had organic matter), and the selling of vegetables beside raw meat or raw fish. Additionally, visual assessments on bathroom facilities showed that handwashing stations in the bathroom were not widely available. Moreover, interior of the bathroom facilities in both provinces were not clean, had standing water, and cracks on walls and floors. (Table 2.3)

2.3.2 Province disaggregated results

A higher percentage of female farmers were observed in SR (peri-urban,75%), with fewer farmers in that province using inorganic fertilizers (45%), when compared to BB (rural) (Table

2.4). In contrast to farmers, a higher percentage of male collectors were observed in BB (82%), as compared to SR (40%). Findings for vegetable handling and cleaning practices can be found in Table 2.4. In brief, approximately 66% of farmers in both provinces do not clean vegetables. A higher percentage of BB collectors (90%) do not wash received vegetables in comparison to SR (50%). Furthermore, vegetables are more often transported in cars and open cargo trucks in BB, whereas motorcycles are more often used in SR. However, a higher number of collectors in BB do not clean vehicles before delivering vegetables (54%) as compared to SR (9%). Findings about selling practices revealed that 96% of BB vendors and 82% of SR vendors cleaned their stalls before selling (Table 2.4). A similar percentage of vendors admitted to changing floor tarps whenever they became damaged (62% in BB, 58% in SR), using fabric gloves (60% in BB, 61% in SR), and storing leftover vegetables at room temperature (96% in BB, 94% in SR). Visual assessment data were not disaggregated due to the imbalanced number of vendors observed in each province.

2.3.3 Gender disaggregated result

Gender did not affect the distribution of vegetable-crops cultivated, washing practices of vegetables and the choice of transportation vehicle to the distribution center. Females used more inorganic fertilizer and engaged in more cleaning practices of stalls as compared to males (Table 2.5). Conversely, males preferred to use organic fertilizer (Table 2.5) and stored leftover vegetables at room temperature as compared to female actors (71% and 33% respectively). Both male and female collectors used cars as their primary mode of transportation for delivering vegetables (Table 2.5) and similar percentages did not clean vehicles before delivery (30% males and 37% females). Overall, females represent a higher population of vegetable value-chain actors than males, especially at retail (Figure 2.8).

2.4 Discussion

Overall results suggest that the flow of vegetables in the value-chain follows the information described by Sokhen et al. (2004). The flow of the value-chain remains consistent as findings from this study suggest a close relationship between actors in the value-chain (i.e. distributors always purchase from the same collectors, etc.). Distribution of vegetable production is also consistent with existing literature, in which leafy greens and salad vegetables (i.e. lettuce, cucumbers) are highly produced —as compared to other types of vegetables— on local farms (Sokhen et al., 2004).

Findings revealed that value-chain actors practiced several behaviors that may contribute to cross-contamination at different stages of the value-chain. These practices include the usage of inadequately composted manure, improper harvest intervals after manure application, infrequent washing practices, improper handling of cut vegetables, and unsanitary practices during transport and sales. In terms of soil amendments, findings from the study are inconsistent with past literature, which states that 80% of Cambodian farmers utilize fresh manure (Eliste & Zorya, 2015). Nonetheless, fresh manure and animal waste "compost" were highly used by farmers (33% and 11%, respectively). Evidence from scientific literature suggest the predominant use of swine manure in Cambodian farms (Huynh, Aarnink, Drucker, & Verstegen, 2006). Improperly composted swine manure is known to be a source of critical foodborne pathogens, such as *Salmonella enterica* (Farzan, Friendship, Cook, & Pollari, 2010; Franz & van Bruggen, 2008; Grewal, Sreevatsan, & Michel Jr, 2007). Responses on composting methods were inconsistent, indicating that farmers may not be following specific guidelines on composting. Cambodia adopts Codex Alimentarius texts for food safety, which do not have specific recommendations on

composting procedures; however, guidelines from foreign regulatory bodies (i.e. United States Food & Drug Administration), require that compost must follow specific requirements — for temperature, amount of turns, and aging time and composition—which are not practiced by Cambodian farmers ((United States Food and Drug Administration, 2018)). For this reason, "compost" used by the Cambodian farmers do not meet international recommendations and may support the transfer of pathogens to vegetables (Beuchat & Ryu, 1997; Natvig et al., 2002; Santamaría & Toranzos, 2003). Furthermore, responses on interval between manure application and harvest was inconsistent. Cambodia does not have recommendations for the harvest intervals after the application of fresh manure on vegetable crops (e.g. USDA-National Organic Program rule indicates a minimum of 120 days between manure application and harvest for vegetables in contact with the soil and 90 days for all other crops), which increases the likelihood of pathogens being present in the soil (United States Department of Agriculture, 2012).

Proper washing is not frequently practiced by multiple actors across the vegetable value-chain in both province by either gender. Washing vegetables using proper methods is an effective method for reducing parasitological and bacterial contamination (Abougrain, Nahaisi, Madi, Saied, & Ghenghesh, 2010; Francis, Thomas, & O'beirne, 1999; Jung, Jang, & Matthews, 2014; Natvig et al., 2002). However, it is critical to note that washing will negatively impact vegetable quality after storage and increase post-harvest loss of vegetables, especially when it is not followed by cool storage (Bachmann & Earles, 2000; Francis et al., 1999). Cool storage is not a widespread practice among value-chain actors in Cambodia, primarily due to the limited financial resources and assistance to purchase and operate refrigeration equipment. It is likely that value-chain actors are choosing not to wash vegetable for this reason. More concerningly, a common practice of cutting bruised portions out of vegetables was observed (Francis et al., 1999). This may exacerbate

safety and quality concerns by increasing microbial proliferation and crop respiration (Cortese, Veiros, Feldman, & Cavalli, 2016). For this reason, there is a need for infrastructure development to increase farmers' access to cool storage, as this is important for preventing food safety and food quality defects. Additionally, due to the absence of cool storage, washing in the value-chain might not be an adoptable strategy to optimize food safety. Thus, in the present, the responsibility for washing vegetables rests in the hands of consumers, and this should be effectively communicated. This can be in the form of verbal communication by retailers, consumer training, consumer education and outreach programs, etc., to ensure that consumers understand that the vegetables being sold are not free from contaminants and that they share a responsibility in ensuring the safety of vegetables prior to consumption.

In terms of transport, vegetables are also not being delivered under cool temperatures which further enhances microbial proliferation and spoilage (Cortese et al., 2016). The lack of sanitation of transport vehicles might introduce cross-contamination to the vegetables. Poorly sanitized transport vehicles, especially those that have been used previously to deliver animal products, can provide niches for pathogens and facilitate pathogen transmission to vegetables that are being transported in the same vehicle (Beuchat, 1996; Ramesh, Joseph, Carr, Douglass, & Wheaton, 2002). Although this study did not explore whether animal products were also transported in the same vehicle, the probability that the practice may occur must be considered.

Selling behaviors of vendors in distribution centers and informal markets might also introduce pathogens to vegetables, as well as support cross-contamination between vegetables. Improper sanitation practices of stalls increases the likelihood of survival of foodborne pathogens on non-food surfaces (i.e. *Salmonella enterica*) (Soumet et al., 1999). Assessment results revealed inconsistency with survey findings (i.e. using damaged tarps), which is commonly observed in

multiple consumer behavioral studies on food safety practices (Patil, Cates, & Morales, 2005). The low frequency of handwashing and improper use of gloves (i.e. using fabric gloves, not removing gloves when touching non-food objects, not sanitizing before use) has been linked to multiple outbreaks caused by cross-contamination (Michaels et al., 2004; Montville, Chen, & Schaffner, 2001). Cross-contamination of foodborne pathogens from raw meat and fish sources to vegetables has also been documented in multiple studies (Roesel & Grace, 2014; Tauxe, 2002). The likelihood of contamination is intensified by the low hygiene conditions in the bathroom facilities that are commonly used by vendors. Standing water and cracks on walls can be reservoir of environmental persister (i.e. *Salmonella*) as well as fecal coliforms, which may support the spread of contamination across vendors in the market (Barker & Bloomfield, 2000; Sinclair & Gerba, 2011).

Gender disaggregated findings revealed that male actors are more heavily engaged in on-farm and collection activities. Males were found to engage in on-farm practices that may contribute to contamination of vegetables more often, as compared to female actors (i.e. higher use of organic fertilizer, higher frequency of not properly cleaning stalls, higher frequency of storing vegetables at room temperature). This is consistent with past findings, primarily due to the fact that women are predominantly responsible for household food preparation and purchases (Dosman, Adamowicz, & Hrudey, 2001; Patil et al., 2005). Furthermore, the greater involvement of women in the vegetable value-chain, especially at retail was also previously observed in Cambodia (Genova II, Weinberger, Sokhom, Vanndy, & Yarith, 2006).

Several practices of value-chain actors were identified to be potential contamination points in the vegetable value-chain. Intervention strategies, such as targeted educational training programs, infrastructure development and regulatory control harmonization are encouraged. Training should focus on farmers to educate on proper composting and manure application

methods to prevent introduction of foodborne pathogens to vegetables. Training can also be targeted towards collectors due to their personal relationship with farmers and the role that they play as the bridge between production and retail sector. Collectors can incentivize farmers to follow food safety practices by only purchasing vegetables from farmers that practice proper composting etc. Vendors should also be trained on safe handling of vegetables such as separation of meats and vegetables, proper handwashing etc. in the market. Moreover, training should be oriented towards gender specific findings. For example, females represent the predominant population of value-chain actors, particularly vendors, thus targeting them will create a robust impact on the overall value-chain and increase the effectiveness of the training. Additionally, training must be accompanied with financial assistance to develop infrastructures at distribution centers and markets to support food safety practices (e.g. cost to purchase and operate a refrigerator, clean water systems, etc.). Lastly, regulatory control should be enacted to ensure that routine food safety practices are properly conducted. Regulatory control includes setting guidelines to communicate the expectations of regulatory agencies for value-chain actors and monitoring whether expectations are being followed. Coordination between regulatory and value-chain actors must be harmonized to ensure that good food safety practices are being communicated properly and effectively. The implementation of basic food hygiene practices at each step of the value-chain will promote multi-hurdle food safety measures, and ultimately ensure the reduction or elimination of foodborne pathogens from causing poor public health outcomes within the country. Future studies should focus on investigating vegetable value-chain actor behaviors from other provinces to create a more complete dataset, as well as conducting consumer food safety behavior studies in Cambodia. The information will be a valuable resource for the development of suitable future intervention strategies.

Table 2.1 Detailed selected survey response of value-chain actors (overall)

	Responses	Percentage of respondents
Farmers (*n*=102)	I use the following type of fertilizer for my crops:	
	(a) Inorganic fertilizer	56%
	(b) Fresh manure	33%
	(c) "Composted" animal waste	11%
	I do not clean harvested vegetables due to:	
	a) A visually clean vegetable does not need to be washed	55%
	b) The vegetables will be sold to a different value-chain actor that will clean the vegetables	19%
	c) The fear of spoilage from washing	13%
	d) Customers asked me to not wash the vegetables	3%
Collectors (*n*=21)	I do not clean received vegetables due to:	
	(a) The belief that the vegetables had been washed by previous actors (e.g. local farmers, export distributor)	47%
	(b) A visually clean vegetable does not need to be washed	33%
	(c) The fear of spoilage from washing	20%
	If I see bruises and cuts on my received vegetables, I will:	
	(a) Discard the vegetables	55%
	(b) Return the vegetables	25%
	(c) Sell the vegetables as they are	10%
	(d) Cut the bruised part and sell the vegetables	10%
	My mode of transportation for delivering vegetables is:	
	(a) Car	36%
	(b) Motorcycle	20%
	(c) Cart that is attached to a motorcycle	20%
	(d) Truck with open cargo	16%
	(e) Truck or car with refrigeration unit	8%
Distributors (*n*=30)	I do not clean received vegetables due to:	
	(a) A visually clean vegetable does not need to be washed	92%
	(b) The belief that the vegetables had been washed by previous actors (e.g. local farmers, export distributor)	8%
	If I see bruises and cuts on my received vegetables, I will:	
	(a) Cut the bruised part and sell the vegetables	60%
	(b) Discard the vegetables	30%
	(c) Return the vegetables	10%
	Vegetables are transported to the distribution center using:	
	(a) Car	68%
	(b) Truck with open cargo	23%
	(c) Motorcycle	6%
	(d) Cart attached to a motorcycle	3%
	I cleaned my stall area before selling vegetables by:	
	(a) Sweeping the stall using a broom	73%
	(b) I don't clean because the stall already looks clean	15%
	(c) Wiping the stall with water	8%

	(d) Wiping the stall with water and soap/disinfectant	4%
Vendors (*n*=100)	I do not clean received vegetables due to:	
	(a) A visually clean vegetable does not need to be washed	64%
	(b) The belief that the vegetables had been washed by previous actors	19%
	(c) The fear of spoilage from washing	17%
	If I see bruises and cuts on my received vegetables, I will:	
	(a) Discard the vegetables	52%
	(b) Cut the bruised part and sell the vegetables	28%
	(c) Return the vegetables	16%
	(d) Sell the vegetables as they are	4%
	Vegetables are transported to the informal market using:	
	(a) Motorcycle	58%
	(b) Cart attached to a motorcycle	24%
	(c) Car	13%
	(d) Truck with open cargo	5%
	I cleaned my stall area before selling vegetables by:	
	(a) Sweeping the stall using a broom	44%
	(b) Wiping the stall with water	34%
	(c) I don't clean because the stall already looks clean	12%
	(d) Wiping the stall with water and soap/disinfectant	10%

Table 2.2 Detailed visual assessment observations on vendors (*n*= 52 vendors: *n*=15 BB and *n*=37 SR)

Visual assessment questions	Overall vendors (%)	BB vendors (%)	SR vendors (%)
What is the vendor set-up?			
(a) On the ground with tarp	37%	20%	43%
(b) Off the ground with table and tarp	63%	80%	57%
Does the tarp show signs of damage (i.e. holes, frayed ends)?			
(a) Yes	95%	0%	95%
(b) No	5%	100%	5%
Does vendor wash hands after touching money or non-vegetable objects?			
(a) Yes	0%	0%	3%
(b) No	100%	100%	97%
Are vendors using gloves?			
(a) Yes	3%*	7%	5%
(b) No	94%	93%	95%
Does vendor remove gloves after touching money or non-vegetable objects?			
(a) Yes	0%	0%	0%
(b) No	100%	100%	100%
Are there signs of pests (i.e. rodents, insects) in the stall vicinity?			
(a) Yes	96%	100%	95%
(b) No	4%	0%	5%
Are there handwashing stations around the stall?			
(a) Yes	52%	7%	70%
(b) No	48%	93%	30%
Are there stalls that sell raw meat or fish in adjacent to the vegetables?			
(a) Yes	52%	53%	51%
(b) No	48%	47%	49%

*All gloves observed were reusable gloves

Table 2.3 Detailed visual assessment observations on markets' bathroom facilities (*n*= 4 bathrooms: *n*=2 BB and *n*=2 SR)

Visual assessment questions	**Overall markets (%)**	**BB markets (%)**	**SR markets (%)**
Is the restroom's interior clean upon first impression?			
(a) Yes	0%	0%	0%
(b) No	100%	100%	100%
Is there a handwashing station in the restroom?			
(a) Yes	25%	0%	50%
(b) No	75%	100%	50%
Are the walls and floors of the bathroom free of cracks?			
(a) Yes	0%	0%	0%
(b) No	100%	100%	100%
Is the floor of the restroom dry and free of standing water?			
(a) Yes	0%	0%	0%
(b) No	100%	100%	100%

Table 2.4 Detailed selected survey responses of value-chain actors (province disaggregated)

	Responses	Percentage of respondents	
		BB (rural)	**SR (peri-urban)**
Farmers BB (*n*= 50) SR (*n*=52)	I use the following type of fertilizer for my crops:		
	a) Inorganic fertilizer	70%	45%
	b) Fresh manure	22%	41%
	c) "Composted" animal waste	8%	14%
	I do not clean harvested vegetables due to:		
	(a) A visually clean vegetable does not need to be washed	52%	59%
	(b) The vegetables will be sold to a different value-chain actor that will clean the vegetables	27%	29%
	(c) The fear of spoilage from washing	15%	12%
	(d) Customers asked me to not wash the vegetables	6%	0%
Collectors BB (*n*= 11) SR (*n*=10)	I do not clean received vegetables due to:		
	(a) The belief that the vegetables had been washed by previous actors (e.g. local farmers, export distributor)	40%	60%
	(b) A visually clean vegetable does not need to be washed	40%	20%
	(c) The fear of spoilage from washing	20%	20%
	If I see bruises and cuts on my received vegetables, I will:		
	(a) Discard the vegetables	63%	60%
	(b) Return the vegetables	13%	30%
	(c) Sell the vegetables as they are	0%	10%
	(d) Cut the bruised part and sell the vegetables	25%	0%
	My mode of transportation for delivering vegetables is:		
	(a) Car	51%	23%
	(b) Motorcycle	8%	31%
	(c) Cart that is attached to a motorcycle	8%	31%
	(d) Truck with open cargo	25%	8%
	(e) Truck or car with refrigeration unit	8%	7%
Vendors BB (*n*= 46) SR (*n*=54)	I do not clean received vegetables due to:		
	(a) A visually clean vegetable does not need to be washed	78%	61%
	(b) The belief that the vegetables had been washed by previous actors	19%	26%
	(c) The fear of spoilage from washing	3%	13%
	If I see bruises and cuts on my received vegetables, I will:		
	(a) Discard the vegetables	61%	44%
	(b) Cut the bruised part and sell the vegetables	5%	48%
	(c) Return the vegetables	32%	4%
	(d) Sell the vegetables as they are	2%	4%
	Vegetables are transported to the informal market using:		
	(a) Motorcycle	79%	38%
	(b) Cart attached to a motorcycle	21%	27%
	(c) Car	0%	25%
	(d) Truck with open cargo	0%	10%

	I cleaned my stall area before selling vegetables by:		
	(a) Sweeping the stall using a broom	47%	46%
	(b) Wiping the stall with water	36%	27%
	(c) I don't clean because the stall already looks clean	4%	19%
	(d) Wiping the stall with water and soap/disinfectant	13%	8%

Table 2.5 Detailed selected survey responses of value-chain actors (gender disaggregated)

	Responses	Percentage of respondents	
		M[1]	F[2]
Farmers M (*n*=30) F (*n*=72)	I use the following type of fertilizer for my crops:		
	a) Inorganic fertilizer	0%	59%
	b) Fresh manure	51%	32%
	c) "Composted" animal waste	49%	9%
	I do not clean harvested vegetables due to:		
	(a) A visually clean vegetable does not need to be washed	66%	50%
	(b) The vegetables will be sold to a different value-chain actor that will clean the vegetables	24%	30%
	(c) The fear of spoilage from washing	5%	17%
	(d) Customers asked me to not wash the vegetables	5%	2%
Collectors M (*n*=13) F (*n*=8)	I do not clean received vegetables due to:		
	(a) The belief that the vegetables had been washed by previous actors (e.g. local farmers, export distributor)	36%	72%
	(b) A visually clean vegetable does not need to be washed	36%	25%
	(c) The fear of spoilage from washing	28%	0%
	If I see bruises and cuts on my received vegetables, I will:		
	(a) Discard the vegetables	58%	50%
	(b) Return the vegetables	17%	38%
	(c) Sell the vegetables as they are	8%	13%
	(d) Cut the bruised part and sell the vegetables	17%	0%
	My mode of transportation for delivering vegetables is:		
	(a) Car	31%	44%
	(b) Motorcycle	19%	22%
	(c) Cart that is attached to a motorcycle	19%	22%
	(d) Truck with open cargo	19%	11%
	(e) Truck or car with refrigeration unit	13%	0%
Distributors M (*n*=9) F (*n*=21)	I do not clean received vegetables due to:		
	(a) A visually clean vegetable does not need to be washed	75%	94%
	(b) The belief that the vegetables had been washed by previous actors (e.g. local farmers, export distributor)	25%	6%
	If I see bruises and cuts on my received vegetables, I will:		
	(a) Cut the bruised part and sell the vegetables	67%	59%
	(b) Discard the vegetables	0%	36%
	(c) Return the vegetables	33%	6%
	Vegetables are transported to the distribution center using:		
	(a) Car	60%	68%
	(b) Truck with open cargo	30%	18%
	(c) Motorcycle	10%	10%
	(d) Cart attached to a motorcycle	0%	5%
	I cleaned my stall area before selling vegetables by:		
	(a) Sweeping the stall using a broom	45%	90%
	(b) I don't clean because the stall already looks clean	44%	5%

	(c) Wiping the stall with water	0%	5%
	(d) Wiping the stall with water and soap/disinfectant	11%	0%

[1]M=Male respondents; [2]F=Female respondents

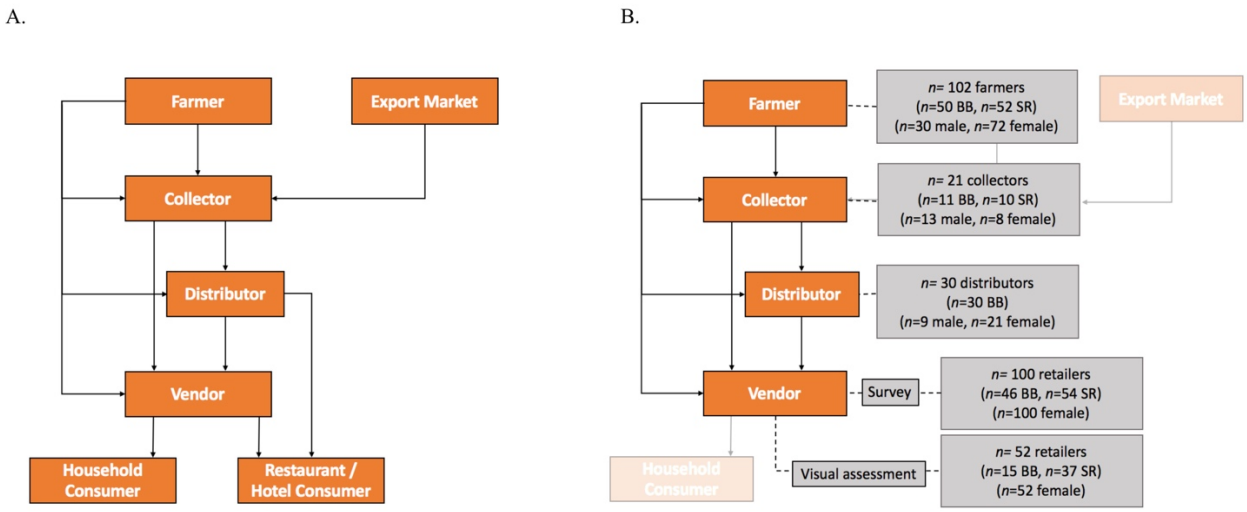

Figure 2.1 A visual schematic of (A) Cambodian vegetable value-chain, as described by Sokhen et al. (2004) and (B) Cambodian vegetable value-chain explored as a part of the current study design

Figure 2.2 Map of Cambodia. Location of data collection: Battambang (rural) and Siem Reap (peri-urban) are highlighted.

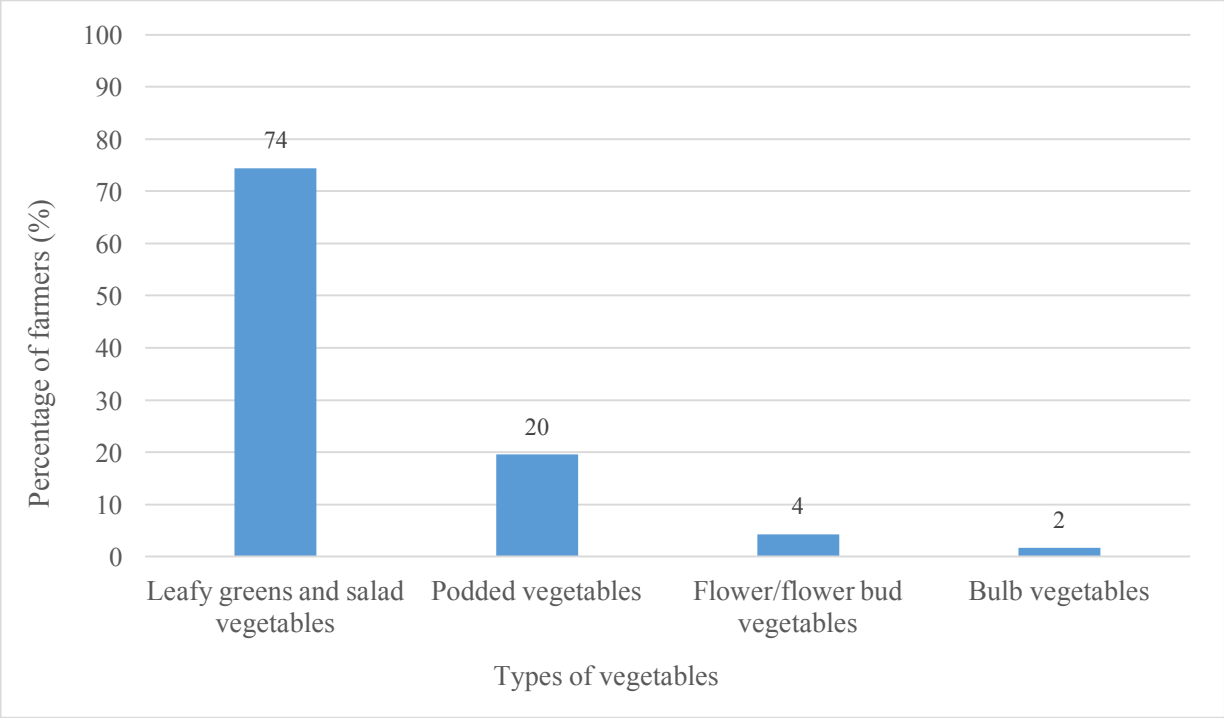

Figure 2.3 Different types of vegetables grown by farmer respondents (*n*=102 farmers)

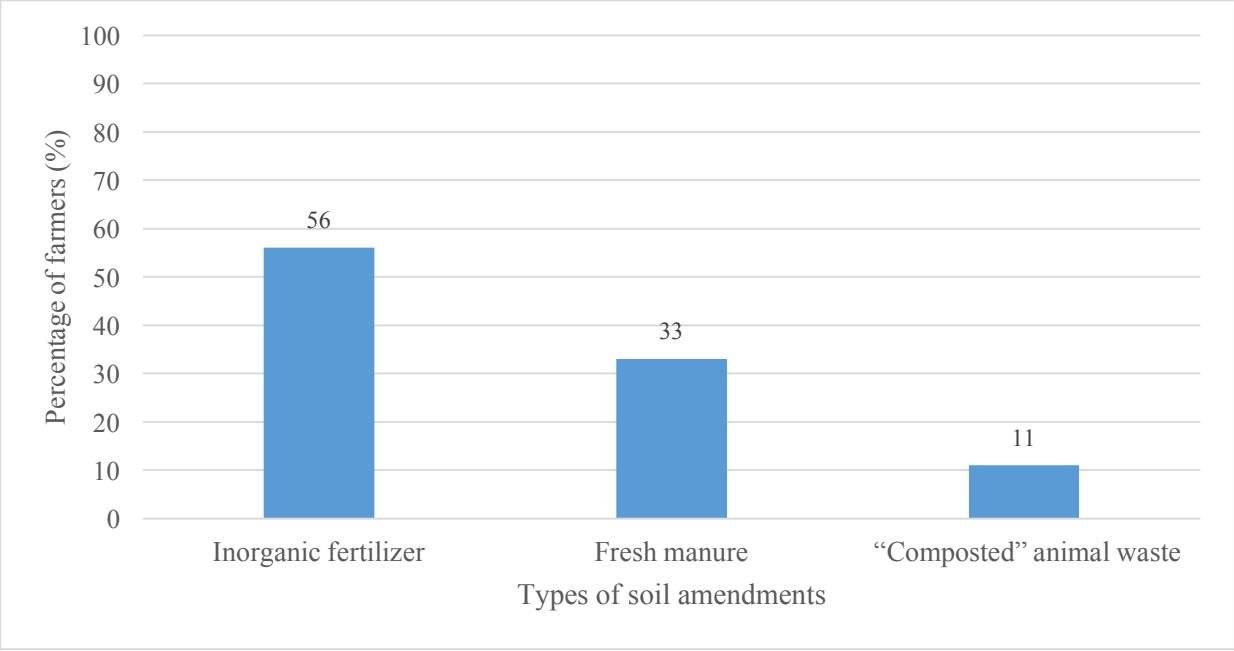

Figure 2.4 Different types of soil amendments used by farmer respondents (*n*=102 farmers)

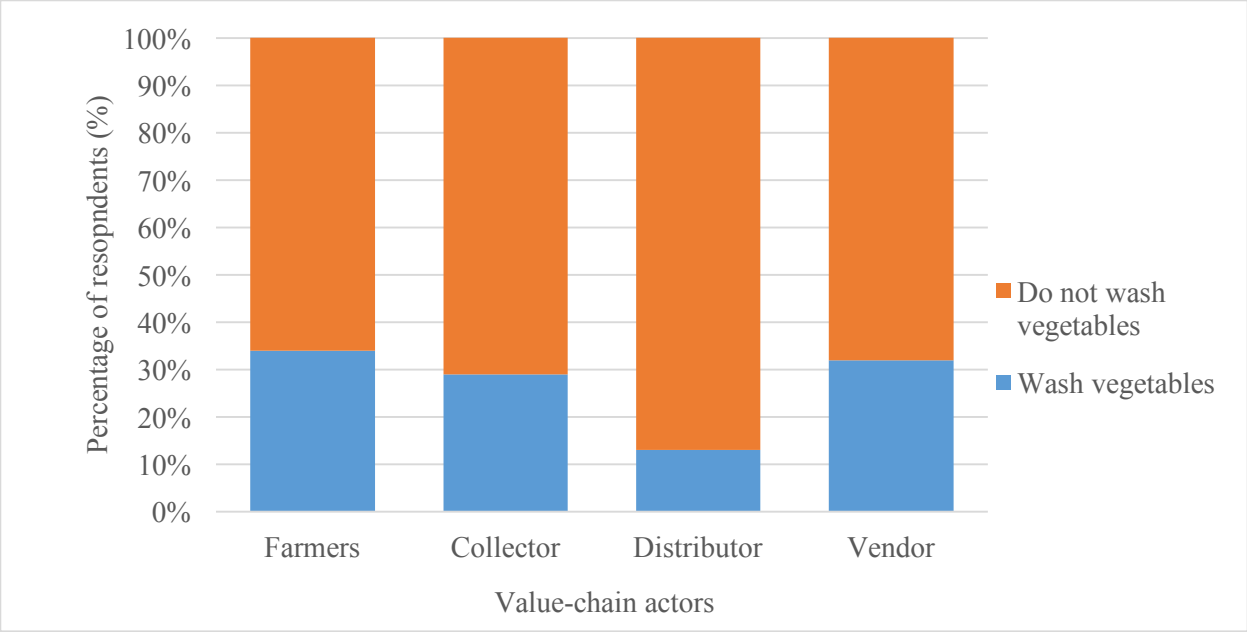

Figure 2.5: Responses on vegetable washing practice by value-chain actor respondents (*n*=102 farmers, *n*=21 collectors, *n*=30 distributors, *n*=100 vendors)

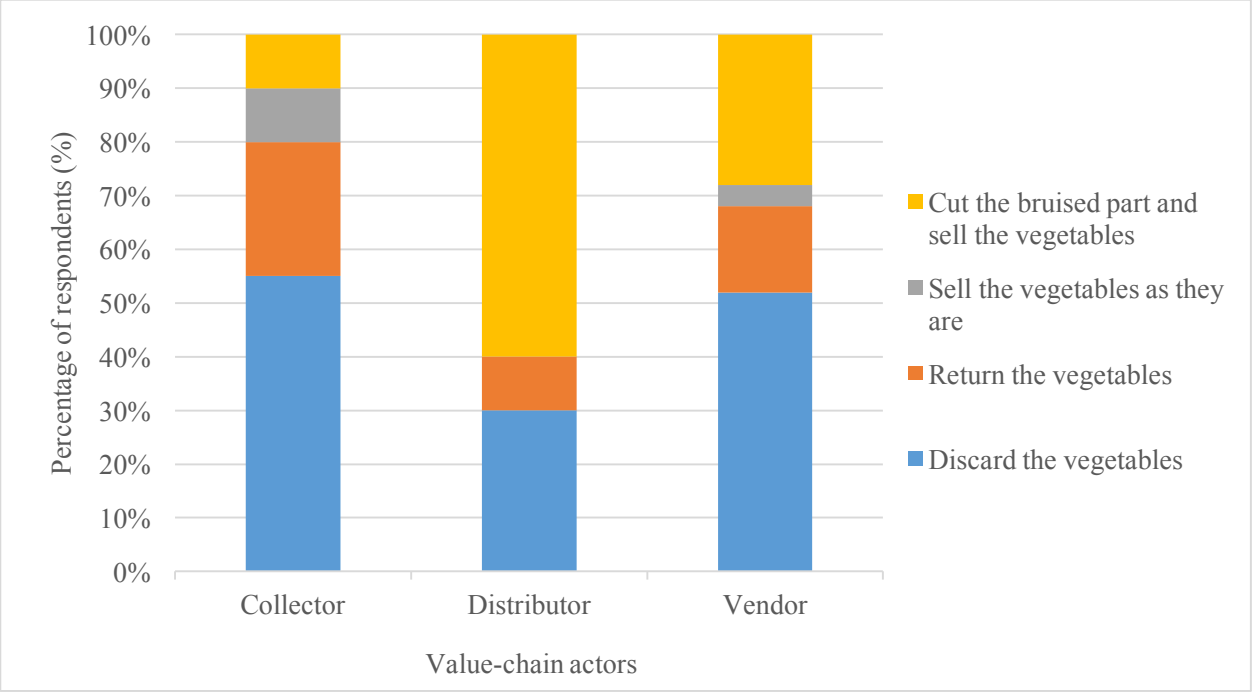

Figure 2.6: Responses on bruised vegetables handling by value-chain actor respondents (*n*=102 farmers, *n*=21 collectors, *n*=30 distributors, *n*=100 vendors)

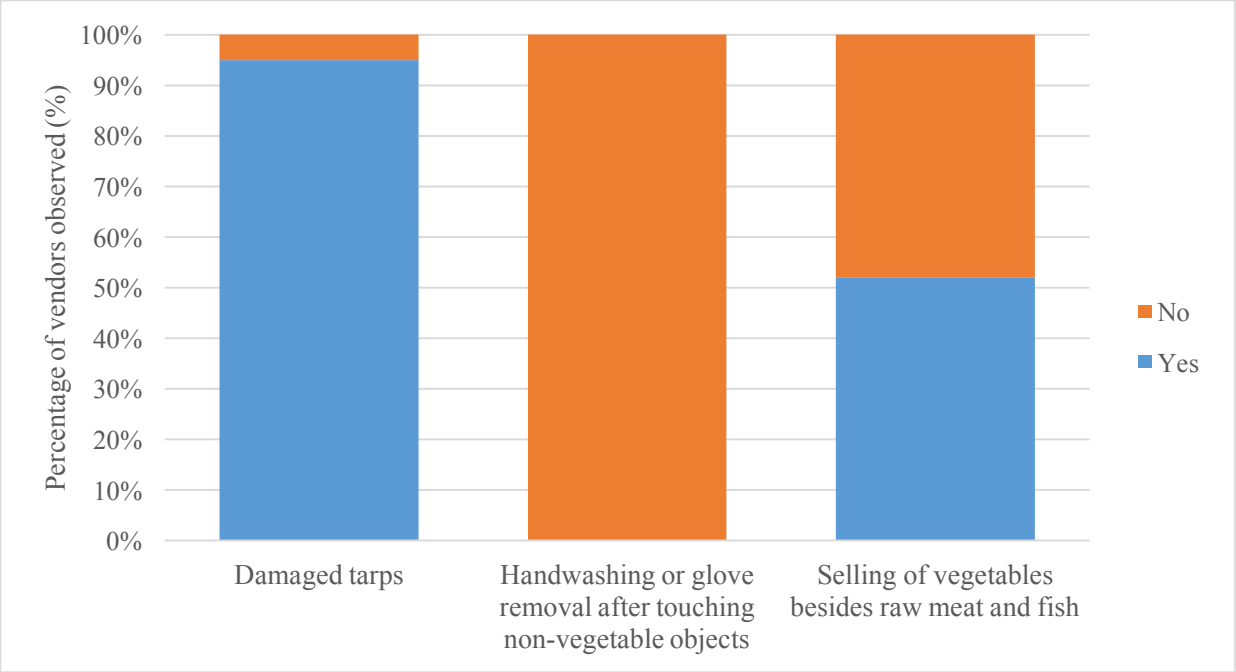

Figure 2.7: Visual assessments of vegetable handling and sanitary practices of vendors in Cambodian informal markets (*n*=52 vendors)

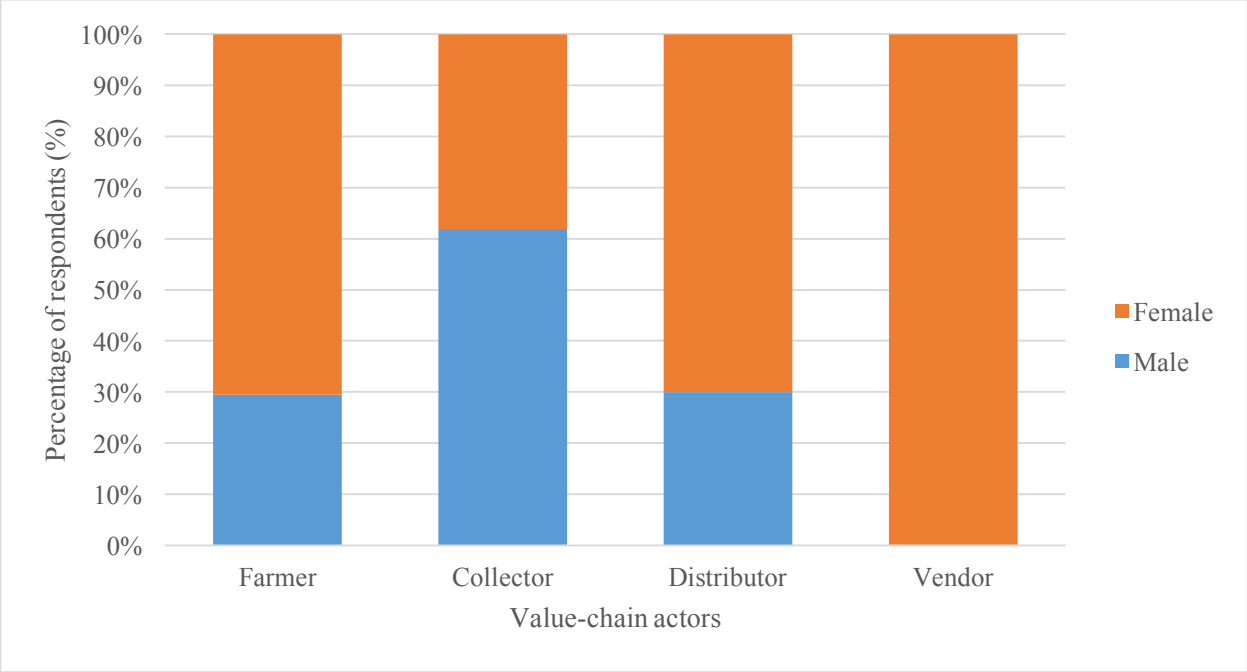

Figure 2.8: Gender demographic of value-chain respondents (*n*=102 farmers, *n*=21 collectors, *n*=30 distributors, *n*=100 vendors)

2.5 References

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Chapter 3 - Prevalence and concentration of *Salmonella enterica*, generic *Escherichia coli* (*E. coli*) and coliforms on fresh vegetables sold in informal markets in Cambodia

3.1 Introduction

Food safety is a key component in securing public health, economic growth, international and domestic trade, and agriculture development in low-and middle-income countries (LMIC) (Grace, 2015; Guerrant et al., 2013; Jaffee et al., 2019). Approximately 600 million foodborne diseases occur globally each year, and evidence shows that unsafe food significantly increases risk of morbidity and mortality in a population, particularly for children below the age of five years (Grace, 2015; Havelaar et al., 2015). Moreover, unsafe food also decreases trading confidence in international markets and threatens domestic markets, resulting in income losses. Income losses include loss of work days, loss of potential due to impaired growth and cognitive development from childhood infection, and loss of productivity due to secondary complications (Guerrant et al., 2013). A recent study estimated that $110 billion (USD) in productivity and medical expenses is lost each year from unsafe food, in LMIC (Jaffee et al., 2019). Finally, unsafe food also has detrimental impacts on vulnerable populations. Vulnerable population are characterized by those that are more susceptible to foodborne illness and those whose financial, social and political abilities are most challenged by the introduction of food safety regulations (Vipham, Chaves, & Trinetta, 2018). For example, pregnant and lactating women are more susceptible to foodborne disease than males, and attempts to modernize food value chains can often result in the exclusion of women from participating (Grace, 2015). Furthermore, food safety programs might unintentionally adopt an “anti-poor” culture because of the increased cost of implementing good

food safety practices, decreasing the affordability of food and access to markets for some of the world's most vulnerable (Vipham et al., 2018). For this reason, food safety programs need to recognize the fundamental differences between social groups, in regards to the values, needs, financial condition and social roles, to create positive impacts in the population. These findings emphasize that food safety has reached multiple areas of a society beyond public health, and its impact is essential on a country's ability to grow and develop.

Southeast Asia, which consists of multiple LMIC, is constantly challenged by food safety issues. This region hosts the highest child mortality rate in the world (Suk et al., 2003). Cambodia—a country in Southeast Asia—struggles significantly with issues related to poverty, with 20% of its population living below the poverty line. This forces a fraction of the country's population to live in unhygienic environments and have limited access to necessities such as safe food and water. In fact, Dasgupta, Deichmann, Meisner, and Wheeler (2005) established a strong correlation between lower income households to inadequate sanitation and child mortality in Cambodia. Child mortality is known to have a profound impact on future productivity of a country (Guerrant et al., 2013; Hoddinott, Alderman, Behrman, Haddad, & Horton, 2013). Lower child mortality lead to an increase in human and country productivity and can support positive development outcomes. In 2018, it was estimated that over 10,000 deaths in Cambodian children occur annually from unknown causes (United Nations Children's Fund, 2018). Historically, diarrheal cases have been attributed to unsafe water, but recent findings suggest that over 40% of diarrheal diseases in developing nations are caused by contaminated food (Food and Agriculture Organization of the United Nations, 2015; Grace, 2015).

The average Cambodian consumes more vegetables and fish than animal-source foods (Sokhen et al., 2004). Therefore, the safety of fish and vegetables have profound effects in

sustaining health and livelihoods of the country's population. A majority of vegetables in Cambodia are sold in informal markets which "escape effective health and safety regulation, are often untaxed and unlicensed" (Roesel & Grace, 2014). These markets are integral to the daily lives of household consumers and vendors, especially women. Women are heavily involved in retail selling of vegetables in the informal markets and depend heavily on its sales for their daily income (Genova II et al., 2006). Although informal markets are central to the lives and culture of people in Cambodia, it is documented that food safety is often neglected in the informal market setting (Roesel & Grace, 2014). This is due to the fact that informal markets lack infrastructural and educational capacity to address food safety issues. For instance, most market vendors do not have tables to display vegetable crops causing them to sell on the ground using tarps. Proper waste disposal, accessible sources of clean water and pest control are also absent. Furthermore, vendors typically have limited education and are not exposed to concepts of food safety. This leads to the lack of proper food hygiene practices in handling vegetable products, such as selling vegetables alongside raw meat and not washing hands after handling non-vegetable objects. The conditions in the informal markets present a food safety challenge and increases the likelihood for the contamination of vegetables, specifically by bacterial hazards.

Pathogenic bacteria are known to be the highest cause of death due to foodborne illness worldwide (Havelaar et al., 2015). Currently, there are limited data on the presence of bacterial hazards on vegetables sold in Cambodian informal markets; however multiple studies support that vegetables sold in informal markets in other Southeast Asian countries (i.e. the Philippines, Vietnam, Thailand, Malaysia) are contaminated with high loads of bacteria, such as *Salmonella enterica* and *Escherichia coli* (*E. coli)* (Ananchaipattana et al., 2012; Chau et al., 2014; Salleh et al., 2003; Pierangeli G Vital et al., 2014). Although Southeast Asian countries are culturally

similar, current data cannot be extrapolated to fit the context of Cambodia— due to differences in socioeconomic status of the country and differences in methodologies used in individual studies. For these reasons, a country-specific study that focuses on the bacterial contamination of vegetables sold in informal markets in Cambodia is needed.

The objective of this study was to investigate the prevalence and concentration of *Salmonella enterica,* as well as the concentration of generic *E. coli* and coliforms—as indicators of cleanliness—on fresh vegetables sold in informal markets in Cambodia. *Salmonella enterica*, specifically non-typhoidal *Salmonella*, has been estimated to cause the highest number of deaths due to foodborne illness on a global scale, creating the largest global burden in comparison to other foodborne disease agent (Havelaar et al., 2015). Moreover, *E. coli* and coliforms are predictors of contamination from fecal origin—a measure of cleanliness and contamination from poor handling of vegetables. This study evaluated the effect of vegetable type as well as seasonality on the prevalence and concentration of *Salmonella enterica*, generic *E. coli* and coliforms. The results from this study helps establish as a baseline data on the presence and levels of contamination in vegetables sold in the Cambodian informal markets. The data will be valuable in the creation of future intervention strategies to mitigate bacterial contamination in these markets, which will potentially contribute to positive public health outcomes in the country.

3.2 Materials and methods

3.2.1 Produce sampling

A total of n=312 fresh vegetable samples were collected from a total of four informal markets in Battambang (n=2) and Siem Reap (n=2) provinces, during two seasons: (1) rainy season (June 2018) and (2) dry season (January 2019) as identified by (Culas & Tek, 2016).Three total

vegetable types were collected: n=104 loose-leaf lettuce, n=104 cucumbers and n=104 tomatoes. These three vegetable types were chosen because they are commonly consumed raw, and have distinct matrices and growing conditions that may contribute to differences in survival and growth of bacterial pathogens. Moreover, seasonal effects were evaluated to identify the effect of temperature and surface run-off from rain on growth and survival of bacterial contaminants. Remote weather data loggers (HOBO MX110 Temp/RH logger) were set up at a location in both Battambang and Siem Reap provinces to measure temperature, humidity and rain fall (Table 3.1). During sample collection days, vegetables were collected randomly from individual vendors in each market, excluding vendors that did not sell all three types of vegetables. Vegetable samples were transferred into separate sterilized, sealable sample bags, labeled, and kept on ice directly after purchasing. Vegetable temperature was monitored using a traceable digital thermometer during transport to the laboratory at Royal University of Agriculture-Phnom Penh. Samples were received at the lab within 48 hours and were immediately analyzed.

3.2.2 Evaluation of *Salmonella enterica*, *E. coli* and coliforms

Sample preparation

Using sterile scalpel blades, 10-gram portions of inner and outer lettuce leaves, tomato skins and cucumber skins were excised from samples, aseptically weighed and transferred to individual filter bags (Nasco Whirl-Pak®, Fort Atkinson, WI-USA). 90 ml of 0.1% Peptone Water (Becton Dickinson, Franklin Lakes, NJ-USA) was aseptically added to each sample bag, and samples were hand-stomached for 1 minute.

Detection and quantification

Salmonella enterica detection methods were adapted from the Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, (Andrews & Hammack, 2007)).

Primary enrichments were created using 1 mL aliquots of sample homogenate, transferred to 9 mL Tryptic soy broth (TSB) (Hardy Diagnostics, Santa Maria, CA-USA) for pre-enrichment incubated at 37° ± 2.0° C for 24 ± 2.0 hours. For selective enrichment, 1 mL of pre-enrichment TSB broth aliquots was transferred to selective enrichment broths of 9 mL Tetrathionate broth (TT) (HiMedia, West Chester, PA-USA) and Rappaport-Vassiliadis (RV) broth (HiMedia, West Chester, PA, USA). Selective enrichments were incubated at 37° ± 2.0° C and 37 ± 2.0° C for 24 ± 2.0 hours respectively. A 1µL loopful of the selective enrichment medium was streaked onto one-third of 100 x 15 mm Y-plate of Xylose Lysine Tergitol-4 (XLT-4) agar and Brilliant green sulfur agar (HiMedia, West Chester, PA, USA). Y-plates were used due to the limited laboratory supplies to accommodate number of samples. Plates were incubated at 37° ± 2.0° C for 24 ± 2.0 hours. After incubation, yellow and clear colonies with dark centers were tested for agglutination using *Salmonella* latex agglutination kit (Oxoid, Hampshire, UK) and recorded as presumptive positives of *Salmonella* enterica

Quantification of *Salmonella enterica* was conducted by following protocols of Brichta-Harhay et al. (2012) and (Webb et al., 2017) with slight modifications. A 1 mL aliquot of sample homogenate was transferred into 9 mL of 0.1% peptone water, creating ten-fold serial dilutions. additional serial dilutions were prepared and were plated onto count plates Enterobacteriaceae (EB) petrifilm™ (3M™, Minneapolis, MN-USA). Count plates were incubated at 37° ± 2.0° C for 24 ± 2.0 hours. Per AOAC Official Methods 2003.01, EB count plates with red colonies associated with entrapped gas, and red colonies with both yellow zones and entrapped gas, were recorded as Enterobacteriaceae. For each EB count plate with characteristic gas producing colonies, the plastic film covers with agar attached was removed from the foam petrifilm™ backing and gently pressed against the surface of an XLT-4 agar plate, replicating the colonies present on the EB count plates.

Plates were incubated at 37° ± 2.0° C for 24 ± 2.0 hours. After incubation, yellow and clear colonies with dark centers were agglutinated using the *Salmonella* latex agglutination kit. Colonies that did not agglutinate or those which morphology atypical for *Salmonella enterica* on XLT-4 were counted and this number was deducted from the total count, to arrive at the count used to estimate the concentration of *Salmonella enterica*. Counts recorded remained as presumptive positives until the detection method of the corresponding sample positively confirmed the presence of *Salmonella enterica*. In instances where a sample was positive but could not be enumerated (i.e. above the limit of detection but below the limit of quantification), a fixed value of of <5 CFU/g (<0.7 $\log_{10}$ CFU/g)—representing half of the limit of detection— was used as the concentration of *Salmonella enterica* for data analysis per EB manufacturer's guideline.

Quantification of *E. coli* and coliforms was conducted by using 1 mL portion of sample homogenate to make ten-fold serial dilutions in 0.1% peptone water. Appropriate dilutions were plated onto *E. coli*/Coliform (ECC) petrifilm™ (3M™, Minneapolis, MN-USA). Count plates were incubated at 37° ± 2.0° C for 24 ± 2.0 hours. Per AOAC Official Methods (998.08 and 991.14), red and blue colonies with entrapped gas were recorded as total coliforms and blue colonies with entrapped gas were recorded as *E. coli*. Count plates were further incubated for another 24 ± 2.0 hours at 37° ± 2.0° C to observe color change. Anytime within the incubation period that a blue colony associated with gas appears, it is recorded as *E. coli.* If total number of colonies were below the recommended countable range for count plates (<15 colonies) on the lowest dilution, a fixed value of <5 CFU/g (<0.7 $\log_{10}$ CFU/g)—representing half of the limit of detection— was used as the concentration of coliforms and *E. coli* for data analysis per ECC manufacturer's guideline.

Salmonella enterica isolates collection

A 1µL loopful of presumptive positive colonies of *Salmonella enterica* were streaked onto 60 x 15 mm plate of Tryptic Soy Agar (TSA) (Hardy Diagnostics, Santa Maria, CA-USA), and incubated at 37° ± 2.0° C for 24 ± 2.0 hours. Isolated colonies were transferred into 9 mL of TSB and further incubated at 37° ± 2.0° C for 24 ± 2.0 hours. The TSB broth was then used to generate a lawn of *Salmonella* growth on TSA using a sterilized cotton swab. TSA plates were incubated at 37° ± 2.0° C for 24 ± 2.0 hours. The lawn was harvested and transferred to individual cryobeads (Key Scientific Products INC, Stamford, TX, USA) by following manufacturer's protocol. Cryobeads were stored at -80° ± 2.0° C until transportation to the United States for isolate confirmation.

PCR confirmation of Salmonella enterica isolates

Confirmation of isolates was completed at Kansas State University. Isolates were activated by transferring a cryobead to 9 mL TSB incubated at 37° ± 2.0° C for 24 ± 2.0 hours. Isolates were prepared using MicroSEQ™ *Salmonella* spp. Detection Kit (with PrepSEQ™ rapid spin sample preparation), per manufacturer's protocol (Thermofisher Scientific, Waltham, MA-USA) and confirmed using Real-Time PCR which target specific target DNA of *Salmonella* spp. (Applied Biosystems 7500 Fast Real-Time PCR).

Statistical Analysis

This study was a randomized complete block design with both vegetable type and season held as the fixed effects. Vendor was set as the blocking factor and was also considered as the random effect. All data were analyzed using SAS 9.4 (SAS Institute, Cary, NC, USA) and Kenward-Rogers adjustment was used to calculate the degree of freedom utilized in the model. LSmeans were separated from all traits of interest by using PDIFF options (F-Test, $p<0.05$)

Prevalence data of *Salmonella enterica* were analyzed using PROC GLIMMIX from SAS 9.4 with estimation achieved by residual pseudo-likelihood method. Over-dispersion term was forced into the model to account for the residual. Quantitative data were log10-transformed and analyzed using the PROC MIXED procedure from SAS 9.4. Levene's test was conducted on quantitative data to ensure homogeneity of variances.

3.3 Results

Vegetables collected from the Cambodian informal markets demonstrated a high prevalence of *Salmonella enterica* contamination. Table 3.2 shows the prevalence of *Salmonella enterica* in the fresh produce. Interactions between vegetable types and season were observed for *Salmonella enterica* prevalence ($P \leq 0.05$, Table 3.2, Figure 3.1). The highest prevalence of *Salmonella enterica* was measured on lettuce collected during the dry season (55.8%), whereas the lowest prevalence was measured on lettuce collected in the rainy season (15.4%). In terms of seasons, rainy season had a significantly lower prevalence of *Salmonella enterica* (18.6%) as compared to the dry season (67.9%; $P \leq 0.05$).

Concentration data of *Salmonella enterica* revealed no significant interaction between vegetable types and seasons ($P=0.2747$), however significant main effects were observed ($P<0.05$) (Table 3.3, Figure 3.2). Lettuce had a significantly higher concentration of *Salmonella* enterica (5.66 $\log_{10}$ CFU/g), as compared to cucumbers (4.20 $\log_{10}$ CFU/g) and tomatoes (3.99 log CFU/g). Further, vegetables in the rainy season (5.27 $\log_{10}$ CFU/g) had a significantly higher counts of *Salmonella enterica* as compared to vegetables in the dry season (3.96 log CFU/g) by more than one log.

Overall, *E. coli* and coliforms were found on 27.2% and 98.7% of total samples, respectively. Concentration data showed a significant interaction between vegetable types and

seasons for generic *E. coli* (P≤0.05) (Table 3.3, Figure 3.3). The highest concentration of *E. coli* was observed on lettuce collected in the rainy season (1.90 $\log_{10}$ CFU/g), whereas the lowest concentration of *E. coli* was observed on cucumber in the dry season (0.81 $\log_{10}$ CFU/g). Similarly, there was a significant interaction between vegetable types and seasons on the concentration of coliforms (P≤0.05) (Table 3.3, Figure 3.4). Lettuce collected in the rainy season demonstrated the highest concentration of coliforms (6.31 $\log_{10}$ CFU/g). This was followed by tomatoes in the rainy season and lettuce in the dry season, which did not differ significantly in concentration of *E. coli* (P=0.77). Furthermore, tomatoes in the dry season had the least concentration of coliforms (3.89 $\log_{10}$ CFU/g). Overall, vegetables collected in the rainy season had a higher concentration of *E. coli* and coliforms as compared to those collected in the dry season. Moreover, lettuce presented the highest concentration of *E. coli* and coliforms as compared to other vegetables.

3.4 Discussion

Fresh vegetables have been increasingly linked to foodborne illness occurrences worldwide (Olaimat & Holley, 2012). In the United States, vegetables were the highest cause of foodborne disease outbreaks from 1990 to 2004, exceeding meats and seafood (Olaimat & Holley, 2012). The consumption of fresh vegetables is also increasingly attributed to diarrhea in Vietnam (Roesel & Grace, 2014). Multiple studies conducted on fresh vegetables sold in the informal markets in Southeast Asia revealed high presence and concentration of foodborne pathogens, particularly *Salmonella enterica* and *E. coli* (Ananchaipattana et al., 2012; Chau et al., 2014; Salleh et al., 2003; Set et al., 2012; P. G. Vital et al., 2014). However, statistical analyses to compare prevalence and concentration data between vegetable types or seasons were not conducted in these studies. Moreover, methodological differences exist between each study which results in data not being comparable between countries.

Historically, *Salmonella* spp. are most commonly linked to poultry; however, recent data suggest that vegetable-related salmonellosis outbreaks are increasing in numbers (Centers for Disease Control and Prevention, 2018b). *Salmonella enterica* can be transmitted to vegetables by cross-contamination with raw meat products as well as non-food environments (i.e. food contact surfaces) (Evans et al., 2003; Soumet et al., 1999). This is pertinent to the conditions in the Cambodian informal markets as vegetables are normally sold alongside raw meat and are exposed to unsanitary environments from vendors selling on the ground. Moreover, basic food hygiene practices are often not practiced in the market environment which increase the likelihood of cross-contamination of *Salmonella enterica*. from environments to the vegetables. Additionally, *Salmonella enterica* are also commonly discovered in swine manure, which are commonly used as fertilizers by Cambodian farmers (Farzan et al., 2010; Grewal et al., 2007; Huynh et al., 2006). Therefore, there is a high likelihood that *Salmonella enterica* contamination could occur during growing, especially when swine manure used for fertilizer is not properly composted or poorly handled. This study shows a high prevalence of *Salmonella enterica* in lettuce (35.6%), with a higher prevalence measured in the dry season as compared to the rainy season (55.8% and 15.4% respectively). This prevalence is much higher when compared to *Salmonella enterica* prevalence in lettuce sold in informal markets in Vietnam (33.3%), the Philippines (30%) and Thailand (5%) (Ananchaipattana et al., 2012; Chau et al., 2014; P. G. Vital et al., 2014). Differences in prevalence might be caused by a difference in methods used to detect *Salmonella enterica* (i.e. Chau et al. (2014) used invA-targeted PCR; P. G. Vital et al. (2014) used Most Probable Methods (MPN); and Ananchaipattana et al. (2012) used biochemical methods. Further, the the studies collected samples in different seasons. Moreover, the current study also revealed that tomatoes from the rainy season had one of the lowest prevalence of *Salmonella enterica* (19.2%), which is consistent

to what was observed in tomatoes from the Philippines (18%) (Pierangeli G Vital et al., 2014). In terms of concentration, the current study revealed that the highest *Salmonella enterica* concentration (average of rainy and dry seasons) was observed in lettuce (5.66 $\log_{10}$ CFU/g). *Salmonella enterica* found in cabbage sold in the informal markets of Cambodia presented a concentration of 3.00 $\log_{10}$ CFU/g. Although these are different types of vegetables, comparisons can still be made as both have similar matrix (Set et al., 2012).

E. coli is commonly linked to produce-related outbreaks due to its association with materials of fecal origins, specifically from manure and poor hygiene of food handlers (Bautista-De León, Gómez-Aldapa, Rangel-Vargas, Vázquez-Barrios, & Castro-Rosas, 2013; Franz & van Bruggen, 2008; Geldreich & Bordner, 1971). Numerous studies have shown that inadequately composted manure and contaminated biological soil amendments may support the transfer of pathogens to vegetables (Beuchat & Ryu, 1997; Natvig et al., 2002; Santamaría & Toranzos, 2003). The predominant use of manure in Cambodia and the absence of following international recommendation for manure handling may increase the likelihood of *E. coli* and other bacteria of fecal origins (i.e. coliforms) to contaminate vegetables (Eliste & Zorya, 2015). Furthermore, the presence of *E.* coli and coliforms on vegetables may also indicate inadequate hygiene practices throughout the vegetable value-chain (Geldreich & Bordner, 1971). For this reason, contamination of vegetables from these bacteria can possibly occur in the informal markets, where poor hygiene has been previously documented (Roesel & Grace, 2014) .The present study detected and enumerated generic *E. coli* and coliforms from vegetable samples collected from informal markets in Cambodia. *E. coli* concentration in lettuce (1.05-2.75 $\log_{10}$ CFU/g) and tomato samples (0.90-1.37 $\log_{10}$ CFU/g) collected in dry and rainy seasons respectively, are lower compared to those observed in lettuce and tomato samples from the Philippines (1.20-3.92 $\log_{10}$ CFU/g in lettuce and

0.88-3.66 $\log_{10}$ CFU/g in tomatoes) as well as in the lettuce samples from Vietnam (5.82 $\log_{10}$ CFU/g) (Chau et al., 2014; Pierangeli G Vital et al., 2014). However, it is important to note that these studies do not evaluate *E. coli* concentration in different seasons. In terms of coliforms, there is currently limited data on its detection and enumeration on fresh vegetables sold in informal markets in Southeast Asia. However, M. Khan, Saha, and Kibria (1992) revealed a coliform concentration range of 2.85-5.76 $\log_{10}$ CFU/g in lettuce and cucumber samples collected in the informal markets in Bangladesh. These levels of coliform contamination are lower than those observed in the present study (5.21-6.31 $\log_{10}$ CFU/g in lettuce, 4.00-4.18 $\log_{10}$ CFU/g in dry and rainy season, respectively).

Overall results showed that lettuce had the highest concentration of all enteric bacteria types evaluated. The susceptibility of lettuce to contamination bybacterial pathogens has been observed previously in multiple foodborne outbreaks, especially those involving *E. coli* O157:H7 in United States (Heiman, Mody, Johnson, Griffin, & Gould, 2015; Wadamori, Gooneratne, & Hussain, 2017). Studies supported that the lettuce matrix provide larger surface area for bacterial attachment (Seo & Frank, 1999; Solomon et al., 2002). Moreover, the stomata and junction zones of cut leaves protect bacteria from wash water and disinfectant (Seo & Frank, 1999). The growing conditions of lettuce also increased the possibility of contact with contaminated manure. In fact, Beuchat (1999) was able to detect pathogenic *E. coli* on manure contaminated lettuce for up to 15 days, even at low inoculum levels. These factors increased the likelihood of pathogen survival and contamination in lettuce. On the contrary, tomato and cucumber samples were observed to have lower concentrations of bacteria as compared to lettuce, which could possibly be due to the smooth pericarp that may inhibit attachment of microorganism (Pierangeli G Vital et al., 2014).

Seasonal differences were also observed. Data showed that *Salmonella enterica* prevalence was higher in the dry season (37.8%), as compared to the rainy season (18.6%). This could be due to irrigation practices being used during the dry season. Beuchat (1996) identified contaminated water to be one of the sources of critical foodborne pathogens in produce production. In Cambodia, irrigation water is mainly sourced from nearby surface waters such as river, ponds and tributaries. The current study did not evaluate the microbiological quality of irrigation water in Cambodia; however a study by Widmer et al. (2013) revealed a high concentration of *E. coli* (3.05 $\log_{10}$ CFU/100 mL) from a major river in Cambodia. According to Geldreich and Bordner (1971) *Salmonella enterica* occurrences reached almost 100% in frequency in surface water with *E. coli* concentration above 3 $\log_{10}$ CFU/100 mL. Moreover, Okafo, Umoh, and Galadima (2003) reported higher counts of *Salmonella enterica* in streams used for irrigation water in Nigeria during the dry season, as compared to rainy season. This may support the fact that irrigation practices may be contributing to differences in *Salmonella enterica* contamination between dry and rainy seasons. Once the vegetables are contaminated with irrigation water, *Salmonella enterica* might persist in the roots and leaves of lettuce for up to 63 days (Islam et al., 2004; Semenov, Van Overbeek, & Van Bruggen, 2009). Conversely, concentrations for all three bacteria types were significantly higher in the rainy season, as compared to the dry season (Table 3.3). Warmer temperatures,higher humidity and rainfall during the rainy season may contribute to a favorable environment for bacterial growth (Table 3.1). Conversely, lower moisture environment during the dry season may create greater stress and energy expenditure for bacteria. This result is consistent with studies evaluating seasonal effects on bacterial growth in manure-amended soil and irrigation water (Natvig et al., 2002; Rai & Tripathi, 2007; Semenov, Van Bruggen, Van Overbeek, Termorshuizen, & Semenov, 2007; Semenov et al., 2009). Furthermore, high rainfall may support

movement of bacteria in the environment, increasing areas of contamination in the market, specifically food-contact surfaces (i.e. basket, tarps etc.). The higher likelihood of contamination of food contact surfaces may contribute to higher load of bacteria found on vegetables collected in the rainy season (Schwan, Desiree, & Vipham, unpublished work). Additionally, differences in prevalence and concentration across seasons may be due to the higher presence of imported vegetables sold in the informal markets during the rainy season. Local farms commonly produce lower quantity of vegetables in the rainy season due to lack of farming infrastructure to combat environmental challenge (i.e. flooding etc.). For this reason, there is often an increased supply of imported vegetables from export markets (i.e. Vietnam and Thailand) to meet demand (Sokhen et al., 2004). Imported vegetables might have different growing and handling procedures which might contribute to the bacterial differences observed between the seasons

Vegetable samples collected in informal markets in Cambodia showed high prevalence and concentration of bacterial pathogens. Although this study did not attribute the contamination to a particular source, it is hypothesized that the use of contaminated irrigation water, the use of improperly composted manure and the unsanitary handling of vegetables in the informal markets are some of the factors that contribute to the transmission of pathogen to the vegetables. Interventions such as educational training on basic food safety practices for vegetable value-chain actors (i.e. farmers, informal market vendors, etc.) as well as consumers are needed. Educational training must be accompanied with financial assistance to develop infrastructure in farms and the informal markets (e.g. clean water for irrigation and handwashing, proper waste disposal, pest control etc.). Lastly, coordination between regulatory agencies and informal market vendors must also occur to ensure that food safety practices are being communicated and conducted. The current study provides baseline data on the presence and levels of bacterial contamination on vegetables

sold in the informal markets in Cambodia. Future studies should focus on collecting higher numbers of samples with wider variety of vegetables to create a more complete dataset. Serotyping of *Salmonella enterica* isolates should also be done to initiate epidemiological surveillance in Cambodia. A source attribution study, relating contamination of to a specific source (e.g. food, environment source etc.) will be useful in determining major contamination routes. Finally, a longer study—conducting sample collection that extends for more than one year—should also be done to capture a better representation of seasonality on pathogen prevalence, bacterial growth and survival.

Table 3.1 Weather data in Battambang and Siem Reap on sample collection months during dry and rainy seasons recorded using remote weather data logger (HOBO MX110 Temp/RH logger)

Season	Province	Temperature (°C)	Rainfall (mm)	Humidity (%)
June 2018 (Rainy)	BB	28.4	0.01	81.1
	SR	28.4	0.03	82.1
January 2019 (Dry)	BB	25.6	0.00	74.8
	SR	26.3	0.00	72.4

Table 3.2 Effects of vegetable types and seasons on prevalence of *Salmonella enterica*.

Factors	Number of positives	Prevalence (%)
Vegetable type effect		
Lettuce	37/104	35.6%
Tomatoes	22/104	21.2%
Cucumbers	29/104	27.9%
P value	N/A	0.0752
Seasons effect		
Rainy	29/156	18.6^{x}
Dry	59/156	37.8^{y}
P value	N/A	0.0003
Vegetable types effect and Seasons effect		
Lettuce-Rainy	8/52	$15.4\%^{A}$
Tomatoes Rainy	10/52	$19.2\%^{AB}$
Cucumbers- Rainy	11/52	$21.2\%^{AB}$
Lettuce-Dry	29/52	$55.8\%^{C}$
Tomatoes-Dry	12/52	$23.1\%^{AB}$
Cucumbers-Dry	18/52	$34.6\%^{B}$
P value	N/A	0.0001

^{x,y} Least squares means with different letters are different in the same column (seasons effect) ($P \leq 0.05$).

$^{A-C}$ Least squares means with different letters are different in the same column (vegetables types and seasons interaction) ($P \leq 0.05$).

Table 3.3 Effects of vegetable types and seasons on concentration of *Salmonella enterica*, generic *Escherichia coli* (*E. coli*) and coliforms

Factors	$\log_{10}$ CFU/g *Salmonella enterica*	$\log_{10}$ CFU/g generic *E. coli*	$\log_{10}$ CFU/g coliforms
Vegetable types effect			
Lettuce	5.66[a]	1.90[a]	5.76[a]
Tomatoes	3.99[b]	1.14[b]	4.56[b]
Cucumbers	4.20[b]	1.05[b]	4.09[c]
SEM[1)]	0.215	0.124	0.104
P value	0.0001	0.0001	0.0001
Seasons effect			
Rainy	5.27[x]	1.80[x]	5.25[x]
Dry	3.96[y]	0.92[y]	4.36[y]
SEM	0.193	0.100	0.088
P value	0.0001	0.0001	0.0001
Vegetable types effect and Seasons effect			
Lettuce-Rainy	6.50	2.75[A]	6.31[A]
Tomatoes Rainy	4.40	1.37[B]	5.27[B]
Cucumber- Rainy	4.90	1.29[B]	4.18[C]
Lettuce-Dry	4.80	1.05[B]	5.21[B]
Tomatoes-Dry	3.59	0.90[B]	3.89[C]
Cucumbers-Dry	3.50	0.81[B]	4.00[C]
SEM	0.347	0.178	0.143
P value	0.2747	0.0003	0.0001

[1)]SEM: standard errors of means;
[a,b] Least squares means with different letters are different in the same column (vegetable types effect) ($P \leq 0.05$);
[x,y] Least squares means with different letters are different in the same column (seasons effect) ($P \leq 0.05$).
[A-C] Least squares means with different letters are different in the same column (vegetables types and seasons interaction) ($P \leq 0.05$).

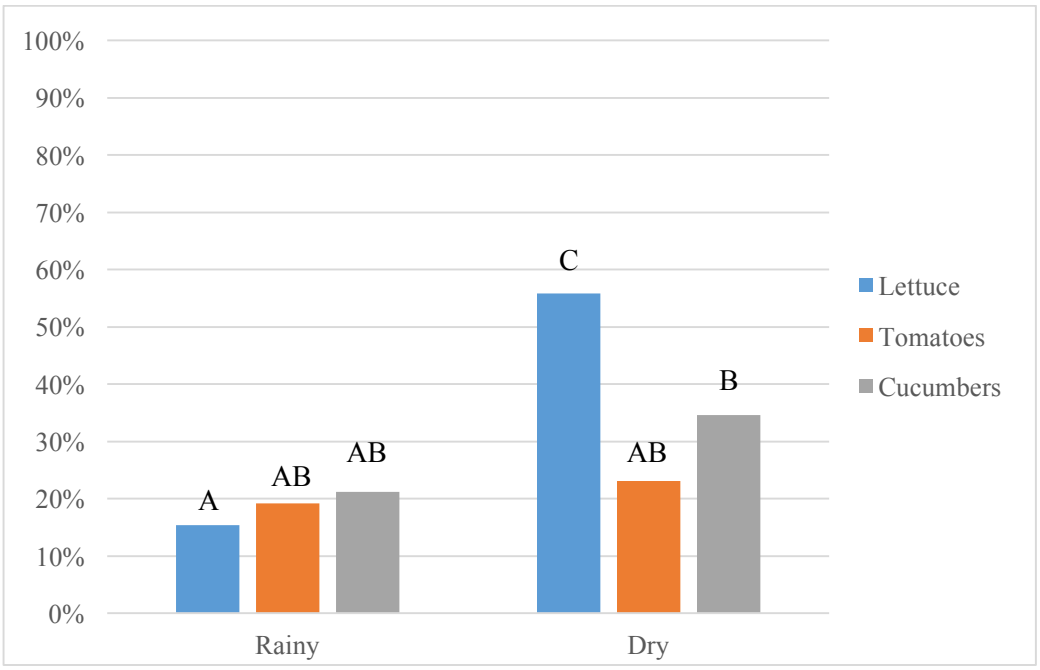

Figure 3.1 Prevalence of *Salmonella enterica* on vegetables sold in Cambodian informal markets collected at two different seasons. Different letters indicate significant differences between groups (P≤0.05).

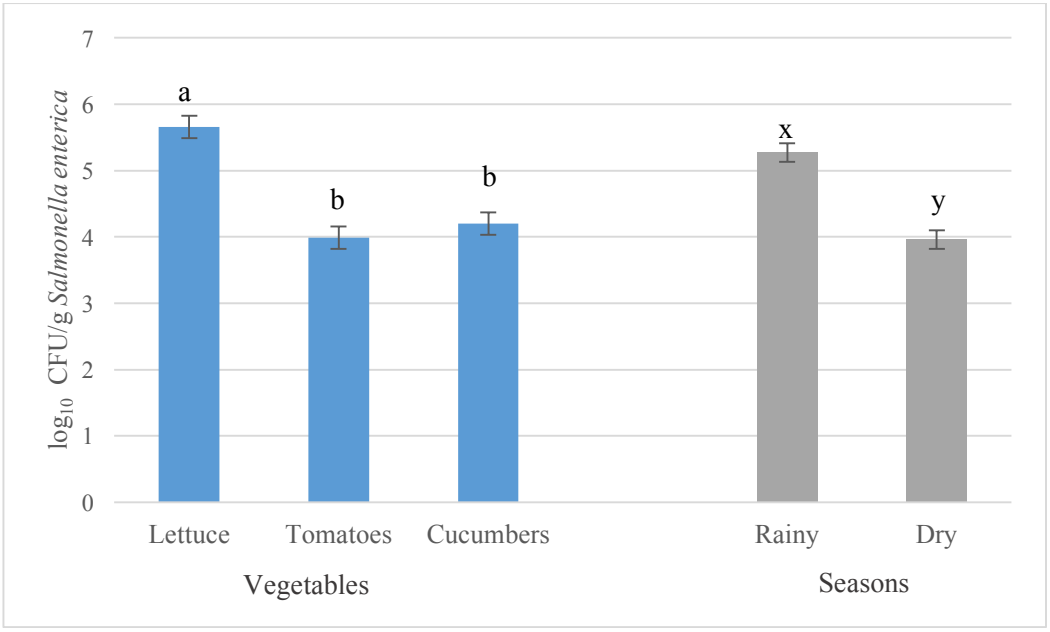

Figure 3.2 Concentration of *Salmonella enterica* on vegetables sold in Cambodian informal markets collected at two different seasons. Different letters indicate significant differences between groups (a,b within vegetables, x,y within seasons) (P≤0.05).

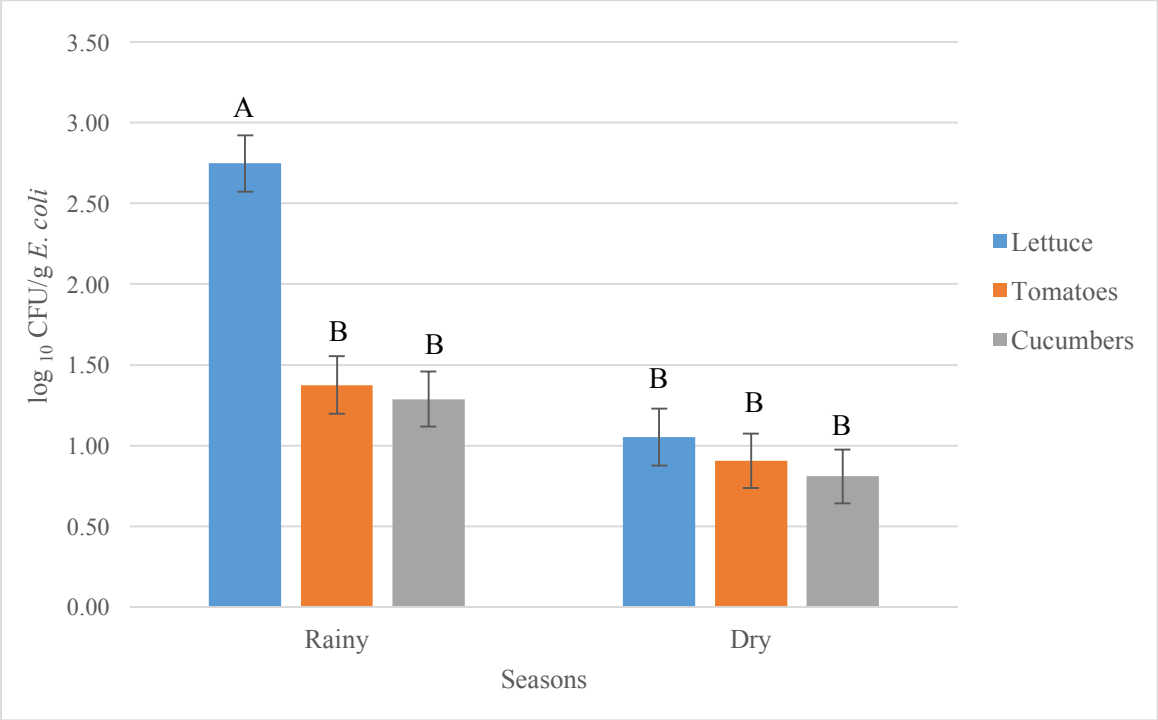

Figure 3.3 Concentration of generic *Escherichia coli* (*E. coli*) on vegetables sold in Cambodian informal markets collected at two different seasons. Different letters indicate significant differences between groups (P≤0.05).

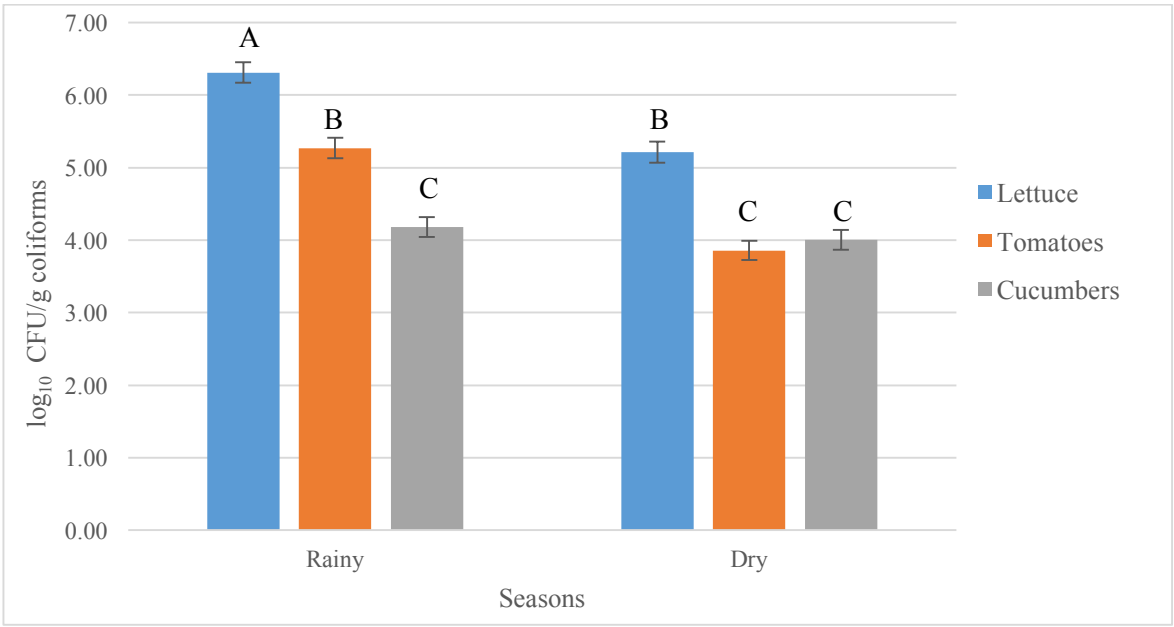

Figure 3.4 Concentration of coliforms on vegetables sold in Cambodian informal markets collected at two different seasons. Different letters indicate significant differences between groups (P≤0.05).

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Chapter 4 - Conclusions

The studies discussed in this document provided insights on the food safety practices and bacterial contamination on vegetables in Cambodia. The study in Chapter 2 identified the flow of the vegetable value-chain in Cambodia, and highlighted some practices that may contribute to contamination of vegetables along the value-chain. The study in Chapter 3 measured bacterial hazards on vegetables (i.e. lettuce, tomatoes and cucumbers) sold in Cambodian informal markets in different seasons. Results from the two studies supported that vegetables sold in informal markets in Cambodia are contaminated with high presence and concentrations of bacteria across seasons. Contamination might occur from poor handling by value-chain actors such as the use of improperly composted manure, the use of unsafe irrigation water, the lack of infrastructure to support food safety practices and the poor hygiene practices of vegetable vendors in the informal market.

The findings from the current study will serve baseline data for the evaluation of biological hazards present in fresh vegetables in Cambodia. However, data generated from this study is a starting point, as gaps are still widely present. First, the current study does not track contamination to a particular source. For this reason, a source attribution study is needed to identify transmission routes of foodborne pathogens in the vegetable value-chain of Cambodia. This can be conducted by analyzing the microbial quality of irrigation water, as well as by analyzing the microbial prevalence and concentration on vegetables at each point of the value chain. Results from a source attribution study will be beneficial in the creation of intervention strategies to address major contamination sources and routes.

Furthermore, the current study solely focused on the hazarde that are present in the Cambodian vegetable value-chain, particularly on vegetables sold in the informal markets.

However, it does not calculate the risk that the hazard might pose to public health. As mentioned previously, risk can be calculated using different measures. It is also heavily impacted by external factors such as social, economic and political factors (Figure 1.2). These factors might introduce actions that would decrease risk (e.g. regulations on safe food handling, food safety training of informal market vendors) or increased risk (e.g. improper handling of vegetables at households, inefficient monitoring of food safety) that would determine the likelihood of an illness to occur. Risk calculation involves other measurements, in addition to measuring the hazard. Thus, further directions should focus on measuring these risk factors that exist in Cambodia, to be able to depict a more complete picture of the food safety status of the country.

Additionally, this study only covered microbial data collection in limited number of vegetables collected from a few markets in two provinces of the country. Survey data was also collected from value-chain actors situated in the corresponding two provinces. Due to this, the data obtained from this study may not provide a complete representation of the country. Further, microbial sample collection was only conducted in a span of one year, with sample collection conducted once in each season. Thus, data in the current study cannot be extrapolated to different years and comparison cannot be made within season. For these reasons, future investments should be made in conducting further microbial and survey data collection with higher number of samples and survey respondents, in more provinces over the span of multiple years. This will help in creating a better representation of bacterial contamination in different seasons and geographic locations in Cambodia.

Lastly, the study conducted in Chapter 2 did not investigate vegetable handling practices beyond retail (i.e. food service and household consumers). Results from this study indicated that consumers in Cambodia may likely be purchasing contaminated produce, and thus must use

proper food safety practices (i.e. washing of vegetables) to reduce their risk. Therefore, future studies should focus on evaluating food safety practices of Cambodian consumers. Results from such a study will be a valuable resource in the creation of consumer education and training programs. This can be part of a multi-hurdle strategy aimed at minimizing the likelihood of bacterial hazards causing poor public health outcome in Cambodia.

Appendix A - Raw data used for statistical analyses

Raw data for prevalence of *Salmonella enterica*

Vendor ID	Seasons	Vegetable	Prevalence
1	Rainy	Lettuce	1
2	Rainy	Lettuce	0
3	Rainy	Lettuce	1
4	Rainy	Lettuce	0
5	Rainy	Lettuce	0
6	Rainy	Lettuce	1
7	Rainy	Lettuce	0
8	Rainy	Lettuce	0
9	Rainy	Lettuce	0
10	Rainy	Lettuce	0
11	Rainy	Lettuce	0
12	Rainy	Lettuce	0
13	Rainy	Lettuce	0
14	Rainy	Lettuce	1
15	Rainy	Lettuce	0
1	Rainy	Tomato	0
2	Rainy	Tomato	0
3	Rainy	Tomato	0
4	Rainy	Tomato	0
5	Rainy	Tomato	1
6	Rainy	Tomato	0
7	Rainy	Tomato	0
8	Rainy	Tomato	1
9	Rainy	Tomato	0
10	Rainy	Tomato	0
11	Rainy	Tomato	0
12	Rainy	Tomato	0
13	Rainy	Tomato	0
14	Rainy	Tomato	1
15	Rainy	Tomato	1
1	Rainy	Cucumber	0
2	Rainy	Cucumber	0
3	Rainy	Cucumber	0
4	Rainy	Cucumber	0
5	Rainy	Cucumber	1
6	Rainy	Cucumber	0

7	Rainy	Cucumber	0
8	Rainy	Cucumber	0
9	Rainy	Cucumber	0
10	Rainy	Cucumber	0
11	Rainy	Cucumber	0
12	Rainy	Cucumber	0
13	Rainy	Cucumber	0
14	Rainy	Cucumber	0
15	Rainy	Cucumber	0
16	Rainy	Lettuce	0
17	Rainy	Lettuce	0
18	Rainy	Lettuce	0
19	Rainy	Lettuce	0
20	Rainy	Lettuce	0
21	Rainy	Lettuce	0
22	Rainy	Lettuce	0
23	Rainy	Lettuce	0
24	Rainy	Lettuce	0
25	Rainy	Lettuce	0
26	Rainy	Lettuce	1
27	Rainy	Lettuce	0
28	Rainy	Lettuce	0
29	Rainy	Lettuce	0
30	Rainy	Lettuce	0
31	Rainy	Lettuce	0
32	Rainy	Lettuce	0
33	Rainy	Lettuce	0
34	Rainy	Lettuce	0
35	Rainy	Lettuce	0
36	Rainy	Lettuce	0
37	Rainy	Lettuce	1
38	Rainy	Lettuce	0
39	Rainy	Lettuce	1
40	Rainy	Lettuce	0
41	Rainy	Lettuce	0
42	Rainy	Lettuce	0
43	Rainy	Lettuce	1
44	Rainy	Lettuce	0
45	Rainy	Lettuce	0
46	Rainy	Lettuce	0
47	Rainy	Lettuce	0
48	Rainy	Lettuce	0
49	Rainy	Lettuce	0

50	Rainy	Lettuce	0
51	Rainy	Lettuce	0
52	Rainy	Lettuce	0
16	Rainy	Tomato	1
17	Rainy	Tomato	0
18	Rainy	Tomato	0
19	Rainy	Tomato	1
20	Rainy	Tomato	0
21	Rainy	Tomato	1
22	Rainy	Tomato	0
23	Rainy	Tomato	0
24	Rainy	Tomato	0
25	Rainy	Tomato	0
26	Rainy	Tomato	0
27	Rainy	Tomato	0
28	Rainy	Tomato	0
29	Rainy	Tomato	0
30	Rainy	Tomato	0
31	Rainy	Tomato	0
32	Rainy	Tomato	0
33	Rainy	Tomato	0
34	Rainy	Tomato	0
35	Rainy	Tomato	0
36	Rainy	Tomato	0
37	Rainy	Tomato	0
38	Rainy	Tomato	0
39	Rainy	Tomato	1
40	Rainy	Tomato	0
41	Rainy	Tomato	0
42	Rainy	Tomato	0
43	Rainy	Tomato	0
44	Rainy	Tomato	1
45	Rainy	Tomato	0
46	Rainy	Tomato	0
47	Rainy	Tomato	0
48	Rainy	Tomato	1
49	Rainy	Tomato	0
50	Rainy	Tomato	0
51	Rainy	Tomato	0
52	Rainy	Tomato	0
16	Rainy	Cucumber	0
17	Rainy	Cucumber	0
18	Rainy	Cucumber	1

19	Rainy	Cucumber	0
20	Rainy	Cucumber	0
21	Rainy	Cucumber	0
22	Rainy	Cucumber	0
23	Rainy	Cucumber	1
24	Rainy	Cucumber	0
25	Rainy	Cucumber	0
26	Rainy	Cucumber	0
27	Rainy	Cucumber	0
28	Rainy	Cucumber	0
29	Rainy	Cucumber	0
30	Rainy	Cucumber	1
31	Rainy	Cucumber	0
32	Rainy	Cucumber	1
33	Rainy	Cucumber	1
34	Rainy	Cucumber	0
35	Rainy	Cucumber	0
36	Rainy	Cucumber	0
37	Rainy	Cucumber	0
38	Rainy	Cucumber	0
39	Rainy	Cucumber	0
40	Rainy	Cucumber	0
41	Rainy	Cucumber	0
42	Rainy	Cucumber	1
43	Rainy	Cucumber	0
44	Rainy	Cucumber	1
45	Rainy	Cucumber	0
46	Rainy	Cucumber	1
47	Rainy	Cucumber	0
48	Rainy	Cucumber	0
49	Rainy	Cucumber	0
50	Rainy	Cucumber	1
51	Rainy	Cucumber	1
52	Rainy	Cucumber	0
1	Dry	Lettuce	1
2	Dry	Lettuce	1
3	Dry	Lettuce	0
4	Dry	Lettuce	0
5	Dry	Lettuce	0
6	Dry	Lettuce	0
7	Dry	Lettuce	0
8	Dry	Lettuce	0
9	Dry	Lettuce	0

10	Dry	Lettuce	1
11	Dry	Lettuce	1
12	Dry	Lettuce	0
13	Dry	Lettuce	1
14	Dry	Lettuce	1
15	Dry	Lettuce	1
1	Dry	Tomato	0
2	Dry	Tomato	1
3	Dry	Tomato	0
4	Dry	Tomato	0
5	Dry	Tomato	0
6	Dry	Tomato	1
7	Dry	Tomato	0
8	Dry	Tomato	0
9	Dry	Tomato	0
10	Dry	Tomato	0
11	Dry	Tomato	0
12	Dry	Tomato	0
13	Dry	Tomato	0
14	Dry	Tomato	0
15	Dry	Tomato	0
1	Dry	Cucumber	1
2	Dry	Cucumber	0
3	Dry	Cucumber	1
4	Dry	Cucumber	1
5	Dry	Cucumber	0
6	Dry	Cucumber	1
7	Dry	Cucumber	0
8	Dry	Cucumber	0
9	Dry	Cucumber	0
10	Dry	Cucumber	0
11	Dry	Cucumber	1
12	Dry	Cucumber	0
13	Dry	Cucumber	0
14	Dry	Cucumber	0
15	Dry	Cucumber	1
16	Dry	Lettuce	1
17	Dry	Lettuce	1
18	Dry	Lettuce	1
19	Dry	Lettuce	0
20	Dry	Lettuce	0
21	Dry	Lettuce	0
22	Dry	Lettuce	1

23	Dry	Lettuce	1
24	Dry	Lettuce	0
25	Dry	Lettuce	0
26	Dry	Lettuce	0
27	Dry	Lettuce	1
28	Dry	Lettuce	1
29	Dry	Lettuce	0
30	Dry	Lettuce	0
31	Dry	Lettuce	0
32	Dry	Lettuce	1
33	Dry	Lettuce	1
34	Dry	Lettuce	1
35	Dry	Lettuce	1
36	Dry	Lettuce	1
37	Dry	Lettuce	0
38	Dry	Lettuce	0
39	Dry	Lettuce	0
40	Dry	Lettuce	1
41	Dry	Lettuce	1
42	Dry	Lettuce	1
43	Dry	Lettuce	1
44	Dry	Lettuce	1
45	Dry	Lettuce	0
46	Dry	Lettuce	1
47	Dry	Lettuce	1
48	Dry	Lettuce	1
49	Dry	Lettuce	1
50	Dry	Lettuce	1
51	Dry	Lettuce	0
52	Dry	Lettuce	0
16	Dry	Tomato	0
17	Dry	Tomato	0
18	Dry	Tomato	0
19	Dry	Tomato	0
20	Dry	Tomato	0
21	Dry	Tomato	1
22	Dry	Tomato	0
23	Dry	Tomato	0
24	Dry	Tomato	0
25	Dry	Tomato	1
26	Dry	Tomato	0
27	Dry	Tomato	1
28	Dry	Tomato	0

29	Dry	Tomato	1
30	Dry	Tomato	0
31	Dry	Tomato	1
32	Dry	Tomato	1
33	Dry	Tomato	0
34	Dry	Tomato	0
35	Dry	Tomato	0
36	Dry	Tomato	0
37	Dry	Tomato	0
38	Dry	Tomato	0
39	Dry	Tomato	0
40	Dry	Tomato	0
41	Dry	Tomato	0
42	Dry	Tomato	0
43	Dry	Tomato	0
44	Dry	Tomato	0
45	Dry	Tomato	1
46	Dry	Tomato	1
47	Dry	Tomato	0
48	Dry	Tomato	0
49	Dry	Tomato	1
50	Dry	Tomato	1
51	Dry	Tomato	0
52	Dry	Tomato	0
16	Dry	Cucumber	1
17	Dry	Cucumber	0
18	Dry	Cucumber	0
19	Dry	Cucumber	0
20	Dry	Cucumber	0
21	Dry	Cucumber	0
22	Dry	Cucumber	0
23	Dry	Cucumber	0
24	Dry	Cucumber	0
25	Dry	Cucumber	0
26	Dry	Cucumber	1
27	Dry	Cucumber	0
28	Dry	Cucumber	0
29	Dry	Cucumber	0
30	Dry	Cucumber	0
31	Dry	Cucumber	0
32	Dry	Cucumber	0
33	Dry	Cucumber	1
34	Dry	Cucumber	0

35	Dry	Cucumber	0
36	Dry	Cucumber	0
37	Dry	Cucumber	0
38	Dry	Cucumber	1
39	Dry	Cucumber	1
40	Dry	Cucumber	0
41	Dry	Cucumber	1
42	Dry	Cucumber	1
43	Dry	Cucumber	1
44	Dry	Cucumber	0
45	Dry	Cucumber	0
46	Dry	Cucumber	1
47	Dry	Cucumber	1
48	Dry	Cucumber	1
49	Dry	Cucumber	0
50	Dry	Cucumber	1
51	Dry	Cucumber	0
52	Dry	Cucumber	0

Raw data for quantitative analysis of *Salmonella enterica*, generic *E. coli* and coliforms

Province	Vendor ID	Vegetable	Coliform (log_{10} CFU/g)	E.coli (log_{10} CFU/g)	Salmonella (log_{10} CFU/g)	Seasons
B	1	Lettuce	5.93	3.65	6.20	Rainy
B	2	Lettuce	6.10	4.65	.	Rainy
B	3	Lettuce	5.75	0.70	6.10	Rainy
B	4	Lettuce	5.80	3.65	.	Rainy
B	5	Lettuce	5.64	0.70	.	Rainy
B	6	Lettuce	6.36	0.70	6.30	Rainy
B	7	Lettuce	5.59	3.95	.	Rainy
B	8	Lettuce	.	.	.	Rainy
B	9	Lettuce	6.59	4.65	.	Rainy
B	10	Lettuce	.	.	.	Rainy
B	11	Lettuce	.	.	.	Rainy
B	12	Lettuce	6.03	0.70	.	Rainy
B	13	Lettuce	6.03	3.65	.	Rainy
B	14	Lettuce	6.46	0.70	6.32	Rainy
B	15	Lettuce	5.65	3.65	.	Rainy

B	1	Tomato	3.14	0.70	.	Rainy
B	2	Tomato	4.57	0.70	.	Rainy
B	3	Tomato	4.59	0.70	.	Rainy
B	4	Tomato	5.95	4.65	.	Rainy
B	5	Tomato	5.66	0.70	5.16	Rainy
B	6	Tomato	5.08	0.70	.	Rainy
B	7	Tomato	5.15	0.70	.	Rainy
B	8	Tomato	.	.	5.39	Rainy
B	9	Tomato	4.80	0.70	.	Rainy
B	10	Tomato	5.13	0.70	.	Rainy
B	11	Tomato	4.77	2.65	.	Rainy
B	12	Tomato	5.64	0.70	.	Rainy
B	13	Tomato	4.75	0.70	.	Rainy
B	14	Tomato	5.01	2.65	4.48	Rainy
B	15	Tomato	5.13	0.70	5.03	Rainy
B	1	Cucumber	3.13	1.65	.	Rainy
B	2	Cucumber	4.12	1.65	.	Rainy
B	3	Cucumber	3.86	0.70	.	Rainy
B	4	Cucumber	1.83	0.70	.	Rainy
B	5	Cucumber	5.17	3.95	5.43	Rainy
B	6	Cucumber	0.70	0.70	.	Rainy
B	7	Cucumber	3.12	1.64	.	Rainy
B	8	Cucumber	4.62	0.70	.	Rainy
B	9	Cucumber	4.13	0.70	.	Rainy
B	10	Cucumber	4.57	0.70	.	Rainy
B	11	Cucumber	2.93	2.60	.	Rainy
B	12	Cucumber	3.50	1.65	.	Rainy
B	13	Cucumber	4.50	0.70	.	Rainy
B	14	Cucumber	4.12	1.65	.	Rainy
B	15	Cucumber	2.93	0.70	.	Rainy
S	16	Lettuce	6.16	0.70	.	Rainy
S	17	Lettuce	5.56	0.70	.	Rainy
S	18	Lettuce	6.84	5.46	.	Rainy
S	19	Lettuce	.	.	.	Rainy
S	20	Lettuce	7.05	4.65	.	Rainy
S	21	Lettuce	5.81	3.62	.	Rainy
S	22	Lettuce	6.50	4.65	.	Rainy
S	23	Lettuce	6.36	0.70	.	Rainy
S	24	Lettuce	7.17	5.63	.	Rainy
S	25	Lettuce	5.41	3.96	.	Rainy

S	26	Lettuce	6.67	0.70	6.40	Rainy
S	27	Lettuce	7.84	6.30	.	Rainy
S	28	Lettuce	7.49	0.70	.	Rainy
S	29	Lettuce	5.52	3.63	.	Rainy
S	30	Lettuce	6.39	4.66	.	Rainy
S	31	Lettuce	5.77	4.12	.	Rainy
S	32	Lettuce	6.65	0.70	.	Rainy
S	33	Lettuce	6.28	0.70	.	Rainy
S	34	Lettuce	6.30	4.65	.	Rainy
S	35	Lettuce	6.35	4.92	.	Rainy
S	36	Lettuce	6.00	0.70	.	Rainy
S	37	Lettuce	6.21	0.70	6.28	Rainy
S	38	Lettuce	6.49	0.70	.	Rainy
S	39	Lettuce	6.59	5.13	6.70	Rainy
S	40	Lettuce	6.71	0.70	.	Rainy
S	41	Lettuce	6.54	6.54	.	Rainy
S	42	Lettuce	6.54	0.70	.	Rainy
S	43	Lettuce	6.28	4.35	6.37	Rainy
S	44	Lettuce	6.67	0.70	.	Rainy
S	45	Lettuce	5.99	4.94	.	Rainy
S	46	Lettuce	6.36	0.70	.	Rainy
S	47	Lettuce	6.50	0.70	.	Rainy
S	48	Lettuce	6.64	0.70	.	Rainy
S	49	Lettuce	6.38	4.95	.	Rainy
S	50	Lettuce	6.29	0.70	.	Rainy
S	51	Lettuce	6.50	5.76	.	Rainy
S	52	Lettuce	6.55	0.70	.	Rainy
S	16	Tomato	4.81	0.70	4.89	Rainy
S	17	Tomato	4.75	0.70	.	Rainy
S	18	Tomato	5.57	0.70	.	Rainy
S	19	Tomato	7.06	6.10	4.65	Rainy
S	20	Tomato	4.72	0.70	.	Rainy
S	21	Tomato	3.84	0.70	4.90	Rainy
S	22	Tomato	7.41	0.70	.	Rainy
S	23	Tomato	5.97	0.70	.	Rainy
S	24	Tomato	4.61	0.70	.	Rainy
S	25	Tomato	.	.	.	Rainy
S	26	Tomato	4.86	0.70	.	Rainy
S	27	Tomato	.	.	.	Rainy
S	28	Tomato	6.45	0.70	.	Rainy

S	29	Tomato	6.11	4.62	.	Rainy
S	30	Tomato	5.21	0.70	.	Rainy
S	31	Tomato	4.86	2.64	.	Rainy
S	32	Tomato	.	.	.	Rainy
S	33	Tomato	4.52	0.70	.	Rainy
S	34	Tomato	5.63	4.43	.	Rainy
S	35	Tomato	5.23	0.70	.	Rainy
S	36	Tomato	.	.	.	Rainy
S	37	Tomato	4.16	0.70	.	Rainy
S	38	Tomato	5.46	0.70	.	Rainy
S	39	Tomato	.	.	0.70	Rainy
S	40	Tomato	5.45	0.70	.	Rainy
S	41	Tomato	5.73	0.70	.	Rainy
S	42	Tomato	5.67	0.70	.	Rainy
S	43	Tomato	7.73	0.70	.	Rainy
S	44	Tomato	5.17	0.70	5.17	Rainy
S	45	Tomato	5.32	0.70	.	Rainy
S	46	Tomato	4.97	0.70	.	Rainy
S	47	Tomato	5.07	0.70	.	Rainy
S	48	Tomato	5.35	2.65	4.97	Rainy
S	49	Tomato	4.85	3.25	.	Rainy
S	50	Tomato	6.09	4.35	.	Rainy
S	51	Tomato	5.32	0.70	.	Rainy
S	52	Tomato	5.00	0.70	.	Rainy
S	16	Cucumber	4.15	0.70	.	Rainy
S	17	Cucumber	3.84	2.55	.	Rainy
S	18	Cucumber	5.51	0.70	5.41	Rainy
S	19	Cucumber	4.01	0.70	.	Rainy
S	20	Cucumber	4.41	0.70	.	Rainy
S	21	Cucumber	4.23	3.13	.	Rainy
S	22	Cucumber	4.55	0.70	.	Rainy
S	23	Cucumber	4.14	0.70	4.51	Rainy
S	24	Cucumber	4.20	0.70	.	Rainy
S	25	Cucumber	3.53	1.65	.	Rainy
S	26	Cucumber	6.85	0.70	.	Rainy
S	27	Cucumber	5.22	0.70	.	Rainy
S	28	Cucumber	4.34	0.70	.	Rainy
S	29	Cucumber	4.46	0.70	.	Rainy
S	30	Cucumber	0.70	0.70	4.42	Rainy
S	31	Cucumber	4.65	0.70	.	Rainy

S	32	Cucumber	5.61	0.70	5.67	Rainy
S	33	Cucumber	4.33	0.70	4.37	Rainy
S	34	Cucumber	3.71	0.70	.	Rainy
S	35	Cucumber	5.31	0.70	.	Rainy
S	36	Cucumber	3.71	0.70	.	Rainy
S	37	Cucumber	4.52	0.70	.	Rainy
S	38	Cucumber	4.47	0.70	.	Rainy
S	39	Cucumber	4.60	2.65	.	Rainy
S	40	Cucumber	5.95	0.70	.	Rainy
S	41	Cucumber	.	.	.	Rainy
S	42	Cucumber	4.58	2.95	4.67	Rainy
S	43	Cucumber	4.38	0.70	.	Rainy
S	44	Cucumber	4.57	4.25	4.57	Rainy
S	45	Cucumber	1.82	0.70	.	Rainy
S	46	Cucumber	4.72	0.70	4.61	Rainy
S	47	Cucumber	4.69	0.70	.	Rainy
S	48	Cucumber	4.35	2.65	.	Rainy
S	49	Cucumber	4.33	0.94	.	Rainy
S	50	Cucumber	4.69	0.70	4.50	Rainy
S	51	Cucumber	5.87	3.65	5.99	Rainy
S	52	Cucumber	4.79	2.65	.	Rainy
B	1	Lettuce	4.78	0.70	4.32	Dry
B	2	Lettuce	4.28	0.70	4.33	Dry
B	3	Lettuce	3.77	0.70	.	Dry
B	4	Lettuce	4.48	0.70	.	Dry
B	5	Lettuce	3.45	2.26	.	Dry
B	6	Lettuce	5.66	0.70	.	Dry
B	7	Lettuce	6.22	0.70	.	Dry
B	8	Lettuce	4.53	0.70	.	Dry
B	9	Lettuce	5.75	0.70	.	Dry
B	10	Lettuce	4.10	0.70	3.43	Dry
B	11	Lettuce	6.29	0.70	6.10	Dry
B	12	Lettuce	5.52	0.70	.	Dry
B	13	Lettuce	4.68	0.70	4.53	Dry
B	14	Lettuce	4.40	0.70	4.57	Dry
B	15	Lettuce	6.31	0.70	5.73	Dry
B	1	Tomato	4.36	0.70	.	Dry
B	2	Tomato	3.84	0.70	3.55	Dry
B	3	Tomato	3.21	0.70	.	Dry
B	4	Tomato	2.84	0.70	.	Dry

B	5	Tomato	2.56	0.70	.	Dry
B	6	Tomato	3.03	1.60	2.49	Dry
B	7	Tomato	4.63	0.70	.	Dry
B	8	Tomato	2.45	0.70	.	Dry
B	9	Tomato	2.82	0.70	.	Dry
B	10	Tomato	4.96	0.70	.	Dry
B	11	Tomato	4.43	0.70	.	Dry
B	12	Tomato	3.49	0.70	.	Dry
B	13	Tomato	1.35	0.70	.	Dry
B	14	Tomato	2.29	0.70	.	Dry
B	15	Tomato	4.85	3.13	.	Dry
B	1	Cucumber	3.93	0.70	1.95	Dry
B	2	Cucumber	2.99	0.70	.	Dry
B	3	Cucumber	4.78	0.70	4.93	Dry
B	4	Cucumber	3.14	0.70	3.13	Dry
B	5	Cucumber	3.88	2.13	.	Dry
B	6	Cucumber	4.31	0.70	3.50	Dry
B	7	Cucumber	0.70	0.70	.	Dry
B	8	Cucumber	3.46	0.70	.	Dry
B	9	Cucumber	5.40	3.95	.	Dry
B	10	Cucumber	2.09	0.70	.	Dry
B	11	Cucumber	4.76	0.70	3.73	Dry
B	12	Cucumber	2.53	0.70	.	Dry
B	13	Cucumber	4.21	0.70	.	Dry
B	14	Cucumber	0.70	0.70	.	Dry
B	15	Cucumber	5.66	0.70	4.35	Dry
S	16	Lettuce	5.59	0.70	5.13	Dry
S	17	Lettuce	5.05	2.95	5.25	Dry
S	18	Lettuce	4.38	0.70	4.27	Dry
S	19	Lettuce	3.71	0.70	.	Dry
S	20	Lettuce	4.25	1.95	.	Dry
S	21	Lettuce	3.75	2.13	.	Dry
S	22	Lettuce	6.68	0.70	4.65	Dry
S	23	Lettuce	.	.	3.30	Dry
S	24	Lettuce	.	.	.	Dry
S	25	Lettuce	.	.	.	Dry
S	26	Lettuce	6.14	0.70	.	Dry
S	27	Lettuce	4.59	0.70	5.13	Dry
S	28	Lettuce	4.61	3.61	5.23	Dry
S	29	Lettuce	4.99	0.70	.	Dry

S	30	Lettuce	5.58	0.70	.	Dry
S	31	Lettuce	6.86	0.70	.	Dry
S	32	Lettuce	3.91	0.70	4.88	Dry
S	33	Lettuce	4.73	0.70	4.93	Dry
S	34	Lettuce	4.73	0.70	4.01	Dry
S	35	Lettuce	4.39	0.70	4.25	Dry
S	36	Lettuce	.	.	0.70	Dry
S	37	Lettuce	.	.	.	Dry
S	38	Lettuce	4.91	0.70	.	Dry
S	39	Lettuce	5.74	0.70	.	Dry
S	40	Lettuce	6.64	0.70	6.08	Dry
S	41	Lettuce	5.52	0.70	5.46	Dry
S	42	Lettuce	5.88	0.70	5.16	Dry
S	43	Lettuce	5.76	0.70	5.24	Dry
S	44	Lettuce	6.55	4.65	5.85	Dry
S	45	Lettuce	5.97	0.70	.	Dry
S	46	Lettuce	5.51	3.95	5.18	Dry
S	47	Lettuce	5.68	0.70	5.48	Dry
S	48	Lettuce	6.39	0.70	6.10	Dry
S	49	Lettuce	6.16	0.70	5.43	Dry
S	50	Lettuce	5.16	0.70	4.65	Dry
S	51	Lettuce	6.22	0.70	.	Dry
S	52	Lettuce	5.26	0.70	.	Dry
S	16	Tomato	4.93	0.70	.	Dry
S	17	Tomato	.	.	.	Dry
S	18	Tomato	4.29	0.70	.	Dry
S	19	Tomato	3.76	0.70	.	Dry
S	20	Tomato	3.88	0.70	.	Dry
S	21	Tomato	4.17	0.70	4.05	Dry
S	22	Tomato	4.70	0.70	.	Dry
S	23	Tomato	2.95	0.70	.	Dry
S	24	Tomato	3.43	0.70	.	Dry
S	25	Tomato	3.23	0.70	3.08	Dry
S	26	Tomato	3.26	0.70	.	Dry
S	27	Tomato	4.20	0.70	3.12	Dry
S	28	Tomato	1.96	0.65	.	Dry
S	29	Tomato	5.13	0.70	4.35	Dry
S	30	Tomato	3.53	0.70	.	Dry
S	31	Tomato	4.57	0.70	3.83	Dry
S	32	Tomato	4.33	0.70	3.55	Dry

S	33	Tomato	4.51	0.70	.	Dry
S	34	Tomato	3.50	0.70	.	Dry
S	35	Tomato	2.10	0.70	.	Dry
S	36	Tomato	2.64	0.70	.	Dry
S	37	Tomato	2.58	0.70	.	Dry
S	38	Tomato	4.13	0.70	.	Dry
S	39	Tomato	5.34	0.70	.	Dry
S	40	Tomato	3.02	1.35	.	Dry
S	41	Tomato	4.36	0.70	.	Dry
S	42	Tomato	4.69	5.05	.	Dry
S	43	Tomato	3.57	0.70	.	Dry
S	44	Tomato	7.69	0.70	.	Dry
S	45	Tomato	4.28	0.70	3.12	Dry
S	46	Tomato	4.81	0.70	3.88	Dry
S	47	Tomato	3.45	0.70	.	Dry
S	48	Tomato	4.91	0.70	.	Dry
S	49	Tomato	5.13	0.70	3.95	Dry
S	50	Tomato	4.42	2.95	4.01	Dry
S	51	Tomato	4.80	0.70	.	Dry
S	52	Tomato	3.59	0.70	.	Dry
S	16	Cucumber	5.54	0.70	5.32	Dry
S	17	Cucumber	3.37	0.70	.	Dry
S	18	Cucumber	3.51	0.70	.	Dry
S	19	Cucumber	1.56	0.70	.	Dry
S	20	Cucumber	4.62	0.70	.	Dry
S	21	Cucumber	3.24	0.70	.	Dry
S	22	Cucumber	3.63	0.70	.	Dry
S	23	Cucumber	2.29	0.70	.	Dry
S	24	Cucumber	5.75	0.70	.	Dry
S	25	Cucumber	4.41	0.70	.	Dry
S	26	Cucumber	4.60	0.70	4.56	Dry
S	27	Cucumber	5.30	0.70	.	Dry
S	28	Cucumber	4.68	0.70	.	Dry
S	29	Cucumber	4.73	0.70	.	Dry
S	30	Cucumber	5.14	0.70	.	Dry
S	31	Cucumber	4.49	0.70	.	Dry
S	32	Cucumber	3.85	0.70	.	Dry
S	33	Cucumber	3.76	1.66	4.15	Dry
S	34	Cucumber	4.65	0.70	.	Dry
S	35	Cucumber	3.42	0.70	.	Dry

S	36	Cucumber	2.76	0.70	.	Dry
S	37	Cucumber	4.37	0.70	.	Dry
S	38	Cucumber	4.40	0.70	3.69	Dry
S	39	Cucumber	5.16	0.70	0.70	Dry
S	40	Cucumber	3.89	0.70	.	Dry
S	41	Cucumber	3.54	0.70	3.11	Dry
S	42	Cucumber	3.58	0.70	3.39	Dry
S	43	Cucumber	4.25	0.70	4.06	Dry
S	44	Cucumber	5.19	0.70	.	Dry
S	45	Cucumber	5.74	0.70	.	Dry
S	46	Cucumber	4.67	0.70	4.31	Dry
S	47	Cucumber	4.59	0.70	3.77	Dry
S	48	Cucumber	4.94	0.70	4.12	Dry
S	49	Cucumber	4.17	0.70	.	Dry
S	50	Cucumber	3.75	0.70	0.70	Dry
S	51	Cucumber	4.61	0.70	.	Dry
S	52	Cucumber	3.36	0.70	.	Dry