Monitoring food safety for rendered fat product handling in the US and the informal vegetable value chain in Cambodia.

by

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Abstract

Food safety is a challenge across all food commodities, and the presence of bacterial pathogens in food is a major public health concern in all countries. In countries with more developed food safety infrastructures, food safety progress is often focused on emerging concerns. For example, it was long thought that finished rendered products were free of biological contamination. However, recent foodborne disease outbreaks associated with rendered products have highlighted the need to better understand pathogen transmission and survival in rendering environments to better eliminate pathogens in such products. The purpose of the first study was to evaluate the effects of moisture levels (0.0%, 0.5%, 1.0%, and 3.0%), storage temperatures (48˚C and 76˚C), and fat characteristics on the growth and survival of *Salmonella enterica* in choice white grease, beef tallow, and chicken fat samples. Samples were inoculated with a high concentration (~10^8 CFU/mL) or low concentration (~10^5 CFU/mL) *Salmonella* cocktail (*S.* Sentfenberg, *S.* Newport, *S.* Thompson and *S.* Infantis) and stored for up to 5 days at 48 ºC or 76 ºC. Remaining populations of *Salmonella* were evaluated daily with and without enrichment step. Bacterial death rates were calculated using the Weibull model for each temperature and moisture level. No significant effect between moisture and/or inoculum level were observed. Only temperature had a significant effect ($P < 0.05$) on *Salmonella* inactivation; when all products were challenged at 76˚C, *Salmonella* concentrations were below detectable limits after 24 hours. At 48˚C a progressive decline in *Salmonella* populations was observed within 3 days in both beef tallow and white grease when samples were inoculated with a high concentration *Salmonella* cocktail. *Salmonella* concentrations were below the detectable limit within 4 days in both fat types when a low concentration cocktail was applied. This research identified the effect of moisture and

temperature in rendered fat samples contaminated with *Salmonella* and underlines the need to use time-moisture-temperature data to minimize microbial growth during transportation and storage.

In low- and middle-income countries, food safety progress may begin with identifying which practices and points along the food chain are of the most concern for public health. Cambodia has introduced several initiatives to increase production and consumption of fresh produce throughout the country. Fresh produce, however, is often associated with foodborne disease; thus, understanding how foodborne pathogens enter and are transmitted throughout Cambodian produce production chains can help to ensure positive nutritional outcomes from increased produce consumption. The second study was conducted to provide a better understanding of transmission of foodborne pathogens throughout vegetable production chains in Cambodia with a focus on the distribution stage by assessing quantitative and qualitative aspects of *Enterobacteriaceae*, coliforms and generic *Escherichia coli* on tomatoes, cucumber, and lettuce sold through a produce distribution center in Battambang, Cambodia. Samples (n = 384) were collected over six-month period spanning (December 2019 - May 2020) and screened for the presence of *Enterobacteriaceae*, coliforms and *E. coli.* Lettuce samples were significantly ($P < 0.05$) more likely to carry *Enterobacteriaceae* and coliforms compared to cucumbers and tomatoes. Additionally, concentrations of *Enterobacteriaceae* were significantly ($P < 0.05$) higher in lettuce (4.71 ± 1.02 $\log_{10}$ CFU/g) samples compared to cucumbers (3.44 ± 1.12 $\log_{10}$ CFU/g), and tomatoes (2.79 ± 1.02 $\log_{10}$ CFU/g). The same trend was observed for coliforms, where lettuce had a significantly higher concentration ($P < 0.05$; 4.36 ± 1.23 $\log_{10}$ CFU/g), followed by cucumber (3.28 ± 1.32 $\log_{10}$ CFU/g) and tomato (2.69 ± 1.45 $\log_{10}$ CFU/g). The results of this study provide an initial assessment *Enterobacteriaceae*, coliforms and *E. coli* contamination in vegetables sold through Cambodian markets. These data can be used to identify sources of

contamination and provide a baseline to assess the efficacy of interventions aimed at limiting contamination and ensuring the safety of fresh produce available to Cambodians.

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Chapter 1 - Literature Review

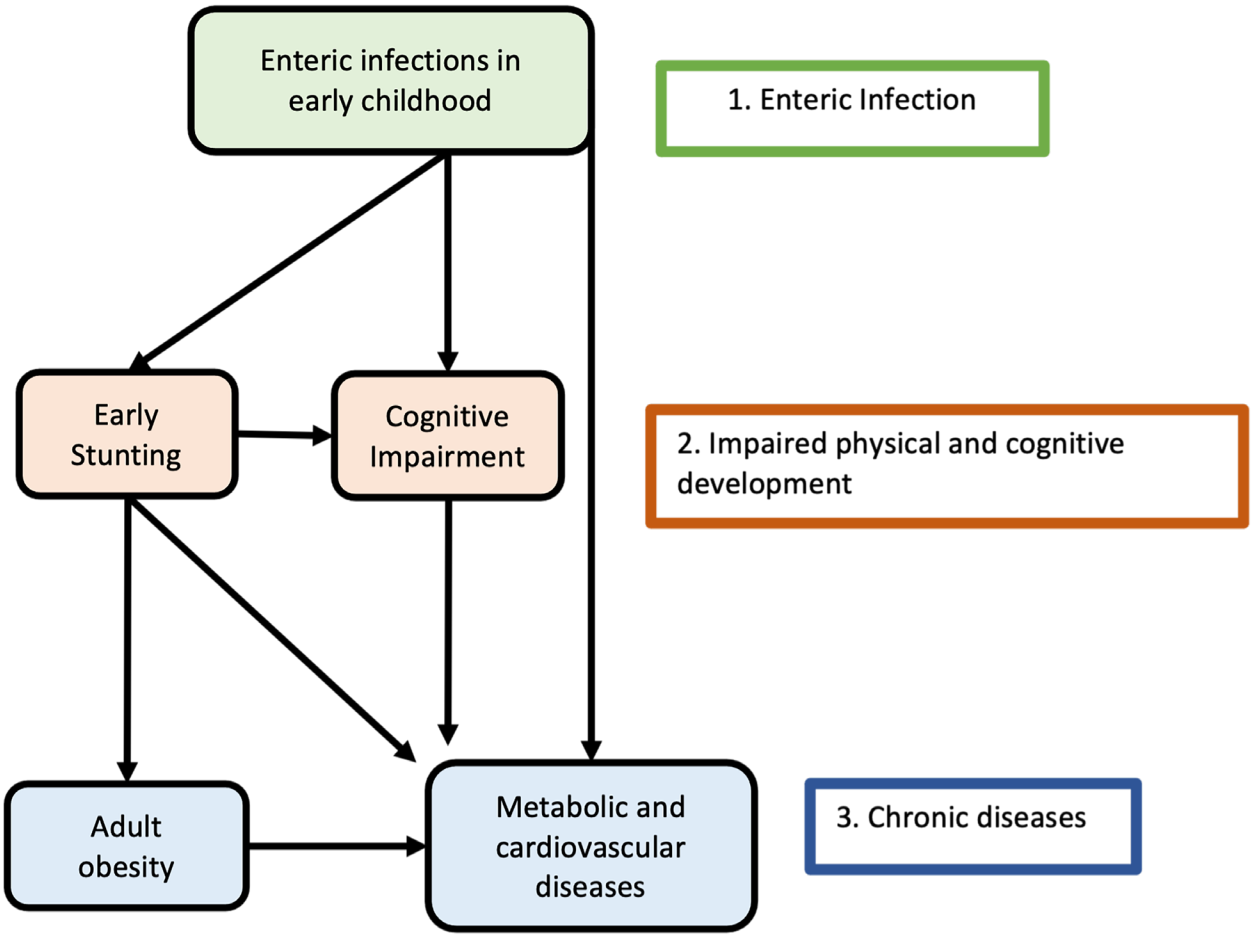
1.1 Public Health Burden and Foodborne Illness

Foodborne disease (FBD), especially during early stages of human development, can result in a wide range of health consequences, including mortality (Guerrant et al, 2013). In non-fatal cases, foodborne diseases can produce both acute (e.g., diarrhea, vomiting, etc.) and chronic or secondary outcomes (e.g., reactive arthritis, stunting, wasting; Buzby, 2001; Lindsay, 1997). While mild cases of FBD may only cause moderate and self-limiting symptoms, severe cases often require hospitalization. Deaths due FBD more commonly occur in the very young, elderly or immunocompromised (Buzby, 2001).

Foodborne diseases are most often associated diarrheal diseases. Guerrant et al, (2013) separates foodborne disease morbidity into three progressive stages, termed the triple burden: 1) enteric infection, 2) impaired physical and cognitive development, and 3) chronic disease (Figure 2; Guerrant et al, 2013). This model emphasizes the long-term effects of FBD including malnutrition, stunting, decreased cognitive function and chronic diseases, such as obesity and cardiovascular disease (Guerrant et al, 2013).

Figure 1.1

The triple burden of diarrheal disease, as described by Guerrant, et al (2013).



In 2015, the World Health Organization (WHO) released estimates on the global burden of FBD caused by 31 foodborne hazards as part of nearly a decade long research effort conducted by the Foodborne Disease Burden Epidemiology Reference Group (FERG) (Havelaar et al., 2015). To quantify the impact of FBD on public health, data is given as both morbidity and mortality estimates (i.e., number of illnesses and deaths, respectively) as well as an estimate of the societal impact of FBD, called disability adjusted life years (DALY). To estimate DALY burden, years lived with disability caused by exposure to FBD and years of life lost due to premature death resulting from FBD are added (Devleesschauwer et al, 2014, Havelaar et al, 2015). Overall, the report found 31 foodborne hazards caused 600 million foodborne illnesses and 420,000 deaths globally in 2010, with 40% of this burden held by children under 5 years of age (Havelaar et al, 2015). The increased risk of FBD for children is due to factors such as underdeveloped immune systems, smaller body size (decreasing the infectious dose needed to induce FBD), and limited control over exposure to food safety risks (Buzby, 2001).

Estimates on the burden of FBD vary greatly between countries and regions (Havelaar et al, 2015). The variation observed can be due to different levels of food safety capacity (i.e., regulatory agency capacity, certification services, and food safety inspection) combined with the country specific data to quantify the countries' burden (i.e., incidence of disease and the related economic costs) (Jaffee et al, 2019). The collective economic impact caused by FBD is due to several factors, such as decreased activity in global markets, loss of trading confidence, cost of medical expenses or income lost while sick (Jaffee et al, 2019). The World Bank (2019) estimated unsafe food collectively costs at least US$110 billion per year in LMIC, which includes both productivity loss (US$95.2 billion) and cost of treating illness (US$15 billion) (Jaffee et al, 2019). A complete understanding of the public health and economic impacts of FBD is important to

motivate research focused on improving food safety. The WHO report on the burden of FBD focused on 31 foodborne hazards (e.g., bacteria, viruses, parasites, toxins, and chemicals) and the majority of burden was contributed to diarrheal disease-causing hazards, such as norovirus, *Campylobacter* spp. and non-typhoidal *Salmonella enterica* (Havelaar et al., 2015).

1.2 Foodborne Disease and Non-typhoidal *Salmonella enterica*

Globally, non-typhoidal *Salmonella enterica* (referred to as *Salmonella*) is a major bacterial pathogen of concern on many levels of public health. According to the WHO, foodborne disease resulted in 230,000 deaths globally in 2010; of which, *Salmonella* contributed approximately 60,000 deaths, the highest total mortality of all foodborne pathogens. Furthermore, diarrheal disease contributed 18 million DALYs to the global burden, with 4 million DALYs contributed by *Salmonella* (Havelaar et al. 2015). *Salmonella* is classified as an agent for both diarrheal and invasive disease and is associated wide range of negative health consequences, including short-term illness, secondary long-term illness, and death (Havelaar et al. 2015, Guerreta et al, 2013). There are more than 2500 *Salmonella* serotypes, all of which are capable of causing FBD (Eng et al, 2015, Jones, et al, 2008). Additionally, *Salmonella* colonization of the host has the potential to spread to the synovial spaces, which leads to the chronic sequelae rheumatoid disease, inflammation of the joints (Lindsey, 1997). This further highlights the significance of *Salmonella* as a microbial pathogen of concern for public health.

Salmonella contains several virulence factors which contribute to transmission and pathogenicity, which may include: flagella, capsule function, plasmids, adhesion systems, and type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity island (SPI)-1 and SPI-2 (Jajere, 2019, Sabbagh, et al, 2010, Shapiro-Ilan, et al, 2005). These virulence factors afford

Salmonella the ability to survive in many food environments and colonize its host through attaching, invading, surviving, and bypassing the host's defense mechanisms (Jajere, 2019). Certain *Salmonella* serotypes, and strains within serotypes, are more associated with certain food products, more frequently cause FBD, or are capable of causing more severe cases of FBD. For example, *S*. Senftenberg has been found to be highly heat tolerant and is frequently associated with slaughter processing facilities (Álvarez-Ordóñez, et al, 2009).

Many foodborne pathogens exist and proliferate in "reservoirs" prior to infecting their host (Prunić et al, 2019). Examples of reservoirs for *Salmonella* include the gastrointestinal tracts of both domestic and wild animals (Percival & Williams, 2013), which become colonized due to presence of *Salmonella* in the environment (e.g., soil, feed, water, plant matter, equipment surfaces; Percival & Williams, 2013, Prunić et al, 2019). In conjunction with environmental factors, high rates of isolation from clinical cases contribute to *Salmonella* being recognized as one of the leading causes of FBD in both developed and developing countries (Havelaar et al. 2015). Due to the frequency with which *Salmonella* is associated with FBD, its presence in food environments is always a food safety concern. *Salmonella* is widely monitored in surveillance systems throughout the world and during outbreaks and is a significant focus of food safety research (McCabe-Sellers & Beattie, 2004, FSIS, 2020). Understanding the main pathogen contributors to public health and economic burden can aid in focusing resources and research efforts on establishing controls for a specific bacteria species, such as *Salmonella*. Therefore, data collected through surveillance and epidemiology can serve as a guide to effective food safety infrastructure placement. Food safety infrastructure includes epidemiological data available to inform food safety decisions, corresponding and responsive governance, infrastructure for laboratory testing and safe food processing, value-chain engagement, and food safety training

(Vipham, Chaves, & Trinetta, 2018). However, if resources are unavailable to generate surveillance and epidemiology data for, such as in a LMIC, food safety issues may not be a priority to governing bodies.

1.3 Indicator Organisms

While there is theoretically a limitless number of organisms able to cause FBD, the majority of FBD outbreaks are the result of smaller group of organisms. In the USA, the top five causes of FBD are Norovirus, *Salmonella, Clostridium perfringens, Campylobacter* and *Staphylococcus aureus* (CDC, 2020). Therefore, process controls in food production facilities should be reliable at eliminating those microorganisms. However, due to restraints such as cost, time, and lab analysis capabilities, it can be impractical to test for specific bacterial agents (Busta et al, 2003).

For surveillance activities, indicator organisms may be used to collect a larger number of samples, save time and resources, be more cost effective or a lower health risk for lab personnel. Indicator organisms may be used to measure the effectiveness of a food safety process control or as an indication of sanitation (Cordier, 2006, Halkman & Halkman, 2014). This works because the conditions in which indicators proliferate are like those of the pathogen of concern (such as optimal growth temperature and nutritional requirements). Depending on the food commodity and production environment, different indicator organisms may be used. Generally, tests for indicator organisms may be a wider family or species of bacteria, in contrast to a specific species or subspecies. In dairy production facilities, *Listeria* spp. may be tested for to monitor the risk of pathogenic *Listeria monocytogenes* (Fairchild & Foegeding, 1993).

The family of *Enterobacteriaceae* includes 10 genera with 20 species being most significant to public health (Rock & Donnenberg, 2014). *Enterobacteriaceae* make up 10% of human and animal intestinal microflora and are their presence in foods considered to be an indicator of potential fecal contamination (Cordier, 2006, Halkman & Halkman, 2014). While the presence of *Enterobacteriaceae* does not indicate an inherent hazard to public health, proper sanitary food handling practices would limit the presence of *Enterobacteriac*eae and other similar, but more pathogenic bacteria (such as *Salmonella enterica*; Cordier, 2006).

Coliforms are a group within the family *Enterobacteriaceae* which include species such as *Citrobacter, Enterobacter, Escherichia*, etc. (Halkman & Halkman, 2014). Like the family *Enterobacteriaceae*, coliforms make up a large percentage of the intestinal microbiome. Thus, there presence in food is another indicator of fecal contamination and unsanitary conditions (Cordier, 2006).

The use of indicator organisms to assess food hygiene is common, especially animal source foods and produce due to concerns over fecal contamination. For example, Carter et al (2021) utilized *Enterobacteriaceae*, coliform, and generic *E. coli* as indicator organisms to evaluate the effectiveness of antimicrobial interventions in beef processing. Similarly, Arthur et al (2004), showed significant correlations between *Enterobacteriaceae* and aerobic plate counts and the presence of *E. coli* O157 H:7 on beef carcasses. (Arthur et al, 2004). Thus, indicator organisms in this scenario allowed for measurement of the efficacy of process lethality of sanitation practices, such as hot water or lactic acid carcass sprays (Arthur et al, 2004, Bosilevac et al, 2006, Carter et al, 2021).

Indicator organisms are also used to indicate the sanitation conditions of produce handling and production environments (Busta et al, 2003, Zoellner et al, 2016, Ruiz-Llacsahuanga et al,

2021). Zoellner et al., (2016) measured the presence of coliforms and generic *E. coli* on tomatoes along the supply chain to better understand microbial dynamics and changes in contamination levels at different locations along the supply chain. Ruiz-Llacsahuanga et al (2021) used aerobic plate count, *Enterobacteriaceae*, coliforms, *E. coli,* and *Listeria* spp as indicator organisms to evaluate the sanitation conditions of an apple packing house. The results supported the use of indicator organisms to rapidly evaluate the efficacy of cleaning and sanitation practices in different production environments. Therefore, indicator organisms can help to advance risk-based approaches to food safety through informing resource allocation.

1.4 Sanitation and Performance Standards to Improve Food Safety

The widespread use of indicator organisms began in 1996, when the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) passed the Pathogen Reduction Final Rule, which established the use of performance standards throughout the animal protein industry (FSIS, 1996). Performance Standards are metrics reported from a food production facility to the government and are used to indicate the effectiveness of processes aimed at controlling (i.e., process controls) pathogens that are of key importance to public health (FSIS, 1996). The goal of performance standards is to use data from product testing to verify the effectiveness of process controls and their ability to ensure the safety of final products available to consumers. The resulting data compiled through the FSIS performance standard program are used to encourage strong relationships with food producers to improve industry wide food safety behaviors (FSIS, 2020). Performance standards also increase attention to sanitation and the practice of documenting food and environmental micro samples. Combined with consumer

pressure to prevent FBD outbreaks, the Pathogen Reduction Final Rule set a precedence for an effective and risk-based food safety system (FSIS, 1996).

In 2020, the FSIS released a document detailing the organization's efforts to reduce *Salmonella* contamination in animal production ("Roadmap to Reducing *Salmonella*"; FSIS, 2020). As part of the revised performance standards, the FSIS provided updated sample testing procedures, which include testing for indicator organisms alongside the pathogen of interest (FSIS, 2020). The goal of widening sampling protocols is to increase verification of controls and increase the proactive approach to preventing FBD.

The risk-based approach to food safety adopted by the USDA FSIS is regarded as one of the most effective strategies to reduce the risk of FBD. Although widespread testing of food production facilities in LMIC is often not feasible, replicating a risk-based approach to food safety may be an effective strategy to improve food safety outcomes. Examples of effective preventative food safety practices include the use of disposable gloves, washing hands frequently, especially after handling money or using restroom, keeping food off the ground and cleaning food-contact surfaces frequently (Jaffee et al, 2019).

1.5 *Salmonella* in Animal Products

The most widely recognized food source associated with salmonellosis is animal sourced proteins. The pathogen colonizes the gastrointestinal tract of many livestock species; thus, mishandling of carcass components or cross contamination with ingesta and fecal material could contaminate edible carcass tissue (Jay, Loessner & Golden, 2005). The association of *Salmonella* and animal proteins was also reflected in a WHO food attribution study that estimated that 75%

salmonellosis cases were associated with the consumption of contaminated animal sourced proteins, primarily eggs, pork, and poultry (Hoffman et al 2017).

In the US, up to one-half of the animal tissue at slaughter does not enter human food systems (Meeker and Meisinger, 2015). Currently, approximately 25 million tons of animal tissue is rendered each year into products such as meat and bone meals and animal fats (Meeker and Meisinger, 2015). In many cases, animal tissues considered inedible or otherwise not fit for human consumption are processed at rendering facilities. Examples of raw material processed at rendering facilities include fatty trimmings, bones, feathers, offal, condemned carcasses and animals that have died outside of a slaughter facility. Due to the nature of tissues that enter into rendering, there are high levels of microbial contamination present. The process of rendering involves chopping products into uniform sizes and cooking them through cycles of 40 to 90 min at 115.6 to 143.3° C in an effort to eliminate all microorganisms present (Meeker and Hamilton, 2006, NRA, 2006). Although the process of rendering is effective for pathogen reduction, cross-contamination between raw material and finished product can occur and is credited as the main contributing factor in reintroduction of *Salmonella* into thermally treated rendered products (Troutt et al., 2001, Kinley, et al 2010, Gong & Jiang, 2017). Handling practices after leaving the rendering facility may also contribute to *Salmonella* harborage, such as residual water in transportation vessels and improper storage temperatures (Trinetta et al., 2019).

The use of rendered products is most common in livestock and pet food industries, which collectively make up 50% of the rendered fat market (Jekanowski, 2011, NRA, 2008). Feed grade fats contaminated with pathogenic bacteria can cause illness in both animals and humans. Humans may become ill after handling contaminated food, interacting with an animal shedding bacterium or through cross contamination. Although low water activity and high fat food matrixes found in

rendered products are thought to create an inhospitable environment for bacteria growth, several recent outbreaks point to a shift in *Salmonella* ability to survive in those conditions. Between 2006-2008, dry dog food contaminated with *Salmonella enterica* serotype Schwarzengrund resulted in 79 reported human illnesses across 21 states. Of those illnesses, 12 required hospitalization and most were children under the age of 2 years old (CDC, 2008). Again in 2012, an outbreak due to dry dog food contaminated with *Salmonella enterica* serotype Infantis sickened 53 people in the U.S. and Canada, 12 of which required hospitalization (Imanishi, 2014). Although the contamination sources for the pet food outbreaks were not identified, use of rendered by-products is a concern due to the practices of applying rendered fat as an outer coating on dry pet food pieces after the thermal cooking step (Thompson, 2008). More research is needed to assess the ability of *Salmonella* to survive in different rendering environments and to identify potential risk factors in post-rendering handling. Understanding these factors could help establish appropriate risk mitigation techniques, such as additional sanitation of food contact equipment and temperature-controlled transportation (Gong & Jiang, 2017, Trinetta et al, 2019).

1.6 Contamination in Vegetables

Fresh produce has increasingly been associated with FBD in the past two decades due to increased consumption, production, distance to distribution along with improvements to surveillance and epidemiology (Olaimat & Holley, 2012). In addition to the risk of contamination at the harvesting stage of production, produce that travels a long distances to the consumer has an increased risk of cross contamination through handling and bulking of produce (Jaffee et al., 2019). In many cases, food safety risks associated with vegetables can be effectively reduced by washing and sanitizing food contact surfaces. However, in low- and middle-income countries (LMIC)

where food safety efforts are new and resources may be limited, there is a need to establish a baseline characterization of contamination that would allow identification of controls and interventions would be most effective.

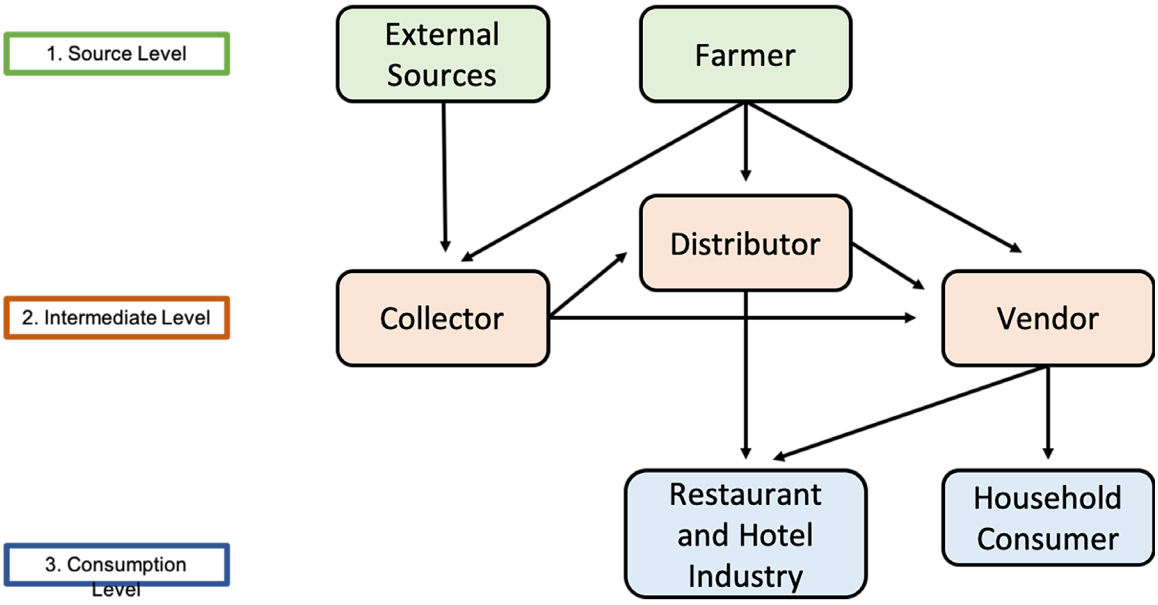
The growth in population size and economies in LMICs is often accompanied by increases in agriculture production, distance food travels to consumer, bulk food storage, lengthening of value chains and changes in diet that may include increased consumption of risky foods (Grace et al, 2015). All of these activities can create new and dynamic food safety challenges. Cambodia has a large agriculture industry with a significant portion (85.6 % of agriculture workers) involving crop production alone (Underhill, 2013). Lack of food safety practices, raw vegetable consumption and a heavy reliance on informal markets which often operate outside of existing food safety regulations could combine to product high levels of risk associated with produce consumption in Cambodia (Olaimat & Holley, 2012, Grace et al, 2015).

An overview of the Cambodia informal vegetable value chain is displayed in Figure 3 and separates food chain actors into three areas: 1) source level; 2) intermediate level; and 3) consumption level (Desiree, 2019, Sokhen et al., 2004). These three areas help to give context to the potential microbial pathogen contamination sources. At the source level, produce can become contaminated due to traditional farming methods, such as using raw manure as fertilizer, poor pest management, improper storage, or other unsanitary practices (Jaffee et al, 2019). At the intermediate level, produce is handled and grouped from several sources for transportation and distribution. Potential sources of microbial pathogens may be introduced through cross contamination, dirty food contact surfaces, poor food handler hygiene, and poor pest control (Jaffee et al, 2019). At the consumption level, food handlers and consumer education should focus on how to source, store and prepare produce to maximize food safety (Jaffee et al, 2019).

There is a great need for research to establish the types, levels, and prevalence of pathogenic bacterial contamination at all levels of the Cambodia vegetable value chain. The task for creating the baseline understanding of microbial contamination in a LMIC such as Cambodia takes multiple years and requires collaboration between academic research institutions, non-government and government organizations. Focusing research efforts on evaluating the dynamics of microbial contamination would allow for better comparisons between the different levels of the vegetable value chain (i.e., source, intermediate and consumption level) and between studies. The use of indicator organisms, such as *Enterobacteriaceae*, coliforms and generic *E. coli,* can provide a cost and time effective approach to establishing the baseline data needed to inform food safety intervention decisions.

Figure 1.2

The flow of produce through the informal vegetable value chain, adapted from Desiree et al, 2020 and Sokhen et al, 2004.



1.7 Conclusions

Improving or establishing risk-based food safety infrastructure in many different food commodities and in production environments is critical for decreasing FBD burden. However, not all countries or food systems face the same food safety challenges. Therefore, it is important to individually assess each food safety problem to determine the best way to approach a solution.

It is clear that *Salmonella* is a significant microbial pathogen of concern. As previously mentioned, recent outbreaks involving rendered products and animal feed have highlighted the need to research how pathogens survive in these food matrixes and how to limit the risk of pathogens in these environments.

In Cambodia, there is a major gap in basic microbial contamination data throughout much of the informal vegetable value chain. This increases the need for region-specific research studies to generate data for informing food safety decisions. When more advanced lab analysis is unavailable or not feasible, indicator organisms play a key role in establishing a baseline understanding of microbial dynamics along a food system or in a food production environment.

This thesis will cover *Salmonella* contamination in rendered fat ingredients in the US and the use of indicator organisms to evaluate produce safety in Cambodia. The specific questions this research addressed were:

- What is the prevalence and concentration of indicator organisms (*Enterobacteriaceae*, coliform and generic *E. coli*) on vegetables (tomato, cucumber and lettuce) sold at the distribution center in Battambang, Cambodia?
- What are the effects of storage conditions (moisture, temperature, time) and fat species (beef, pork, chicken) on *Salmonella* survivability in rendered fat?

Combined, these two studies give an in-depth look at methods to enhance food safety practices in several food commodities and environments. Additionally, these projects assess and observe food safety issues in the context of both developed and developing food safety systems.

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Chapter 2 - Effects of Moisture and Temperature on *Salmonella* Survivability in Beef Tallow, White Grease and Chicken Rendered Fat

2.1 Significance and Impact

Bulk fats and oils used as pellet coatings are rendered free of pathogens in most instances. Nevertheless, they can be re-contaminated with *Salmonella* by handling practices that allow for contaminated contact surfaces and uncontrolled ingress of water from environmental sources and cleaning water. This presents a significant hazard given the application of these fats post the lethality step. The data from the study presented here indicated that holding the fats at temperatures of 76°C or greater resulted in minimal detectable *Salmonella* after 24 hours. At the moisture levels, times and temperatures employed in this study, no pathogen was detected following treatment; lethality was a function of time and temperature. Most rendering operations, however, do not typically hold liquid fats for more than a few hours at a time during peak production times. As such, the time and temperature required for significant pathogen lethality may not be practical in commercial settings yet relying on heat treatment for short periods as practiced in commercial settings may not be adequate to remove *Salmonella* from rendered products.

The data from this study will help end users better understand how these fats should be stored and used to best prevent introducing *Salmonella* into their various products. Further research should focus on identifying more robust ways to eliminate *Salmonella* from the fat matrix either during rendering or further processing.

2.2 Abstract

Rendered fat characteristics, such as water activity and fatty acids composition, may contribute to *Salmonella* survivability. For example, high moisture levels introduced to fats after the rendering process can lead to *Salmonella* growth if present. Limited research, however, is available on strategies to eliminate pathogens in these environments. The purpose of this study was to evaluate the effects of moisture levels (0.0%, 0.5%, 1.0%, and 3.0%), storage temperatures (48˚C and 76˚C), and fat characteristics on the growth and survival of *Salmonella* in beef tallow, white grease and poultry fat samples. Samples were inoculated with a high concentration (~10^8 CFU/mL) or a low concentration (~10^5 CFU/mL) *Salmonella* cocktail (*S*. Sentfenberg, *S*. Newport, *S*. Thompson and *S*. Infantis). Samples were then stored for up to 5 days at 48 or 76 ºC. *Salmonella* was measured in each sample qualitatively (presence; yes/no) and quantitatively (CFU/mL) daily. Death rates were calculated using Weibull model for each temperature and moisture level. No significant effect between moisture and/or inoculum level were observed. Only higher temperature had a significant effect ($P < 0.05$) on *Salmonella* concentrations; when all products were stored at 76˚C, *Salmonella* concentrations were below detectable limits after 24 hours. When products were stored at 48˚C, a progressive decline in *Salmonella* concentrations was observed within 3 day for both beef tallow and white grease when challenged with the high concentrations *Salmonella* cocktail. *Salmonella* concentrations were below detectable limits within 4 days in both fat types when the low concentration *Salmonella* cocktail was instead applied. This research identified that higher storage temperature for rendered fat samples contaminated with *Salmonella* had the greatest impact on reducing populations of the pathogen. These findings underline the need to use time-moisture-temperature data to minimize microbial growth during transportation and storage.

2.3 Introduction

Rendering is defined as the process that converts waste animal tissues into stable and usable materials. It is estimated that the rendering industry collects and processes approximately 25 million tons of animal by-products each year in the United States (Meeker and Meisinger, 2015). Livestock and pet food make up for 50% of the rendered fat market, followed by industrial chemicals, soaps, food industry, and biodiesel (Jekanowski, 2011; NRA, 2008). The most common animal species used in this industry are beef, pork or poultry. Poultry fat consists of fats derived exclusively from poultry offal. Blended feed fat is a category that includes blends of tallow, grease, poultry fat, and restaurant grease/cooking oils. Blended animal and vegetable fats include blends of feed grade animal fats, poultry fats, vegetable fats, and/or restaurant grease/cooking oil.

The process of rendering involves chopping products into uniform sizes and cooking them through cycles of 40 to 90 min at 115.6 to 143.3°C (NRA, 2008). The purpose of this continuous cooking process is to separate useful by-products and eliminate bacteria (Meeker and Hamilton, 2006). Continued improvements within the industry have been implemented to ensure cook times and temperatures inactivate specific microorganisms deemed to be food safety hazards (Meeker and Hamilton, 2006). Despite the thermal processing step, *Salmonella* found in rendered products has been linked to several salmonellosis outbreaks associated with animal and pet food. Cross-contamination, in which finished rendered products become re-contaminated, has been proposed as the primary factor for pathogen presence (Troutt et al., 2001; Denton et al., 2005; Kinley et al., 2010; Vidyarthi, 2021).

The Food and Drug Administration (FDA, 2019) reported that *Salmonella* contamination was responsible for 14.3% of pet food recalls from 2008 to 2014. *Salmonella enterica* serotype Infantis and *Salmonella enterica* serotype Schwarzengrund were identified in the contaminated

dry dog food, causing 53 and 70 illnesses, respectively (Deasy, et al, 2008; Imanishi et al, 2014). In 2018, 22 of the reported pet food recalls were linked to *Salmonella* (FDA, 2019). Though the source of contamination for some of these outbreaks was not determined, the use of rendered products in pet treats formulation was identified as a concern. Rendered by-products are commonly used as an incorporated ingredient and/or an outer coating for dry pet food pieces after the thermal cooking step (Thompson, 2008). The formulation of livestock feeds uses a similar process of adding rendered fats and meals (Crump, 2002). Outbreaks of *Salmonella enterica* which have been traced back to animal feeds, such as poultry and cattle, have identified rendered products as the source of contamination (Crump, 2002).

Some *Salmonella* strains are resistant to desiccation and able survive in low-moisture foods for extended periods of time (Subedi and Roopesh, 2020). Even though low water activity in ingredients such as animal fat, provide a very inhospitable environment for *Salmonella* growth, it is now acknowledged that additions of even small quantities of water to these ingredients may facilitate growth of bacteria. If residual water from wet cleaning of tankers or trucks is accumulated, *Salmonella* spp. can survive and thrive during transportation. Since this ubiquitous pathogen quickly adapts to new environmental conditions (Gwyther et al., 2012), preventing contamination is critical.

The factors and parameters that can influence and favor *Salmonella* growth in low-moisture food, such as animal fat, is important to investigate. Previous data collected by our group (Trinetta et al., 2019) suggested that residual moisture in containers during transportation of poultry fat did not impact *Salmonella* growth. Nevertheless, if the level of *Salmonella* contamination levels were high (~10^8 cfu/mL) and the storage temperature was 48°C, moisture had a role towards pathogen thermal death. In the present study, we measured and compared the effects of moisture levels

(0.0%, 0.5%. 1.0% and 3.0%) and temperatures (48ºC and 76ºC) on the growth and survival of *Salmonella* in different types of animal fat (beef tallow and white grease) overtime.

2.4 Material & Methods

Samples.

Beef tallow, choice white grease, and chicken fat samples were obtained from a local supplier (Manhattan, KS). Rendered fats were transported, received and stored in airtight and opaque containers and held at room temperature.

Physical and chemical properties.

Fat composition was calculated using a chloroform/methanol lipid extraction (Rice, et al., 2019). The fatty acid composition of each rendered fat type was determined at the Kansas Lipidomics Research Center, Kansas State University using fatty acid methyl esters (FAME) method (Christie, 1993). Transmethylation was performed using methanolic hydrochloric acid (3M) at 78°C for 30 min. FAME were extracted using hexane: chloroform (4:1 v/v) mixture, and injected (0.5 µL) to a GC-FID system (Agilent 6890N system). Fatty acid percentages were calculated using corresponding peaks, which were identified based on relative retention times in the FAME standard mix with known concentration. Moisture percentage was calculated by oven drying at 137 ºC for 2 hours (AOAC, 2005; method 930.15). Water activity was measured using a benchtop Aqualab machine (Meter Group, USA).

Microorganisms.

Salmonella enterica Thompson (ATCC 13311), *Salmonella enterica* Newport (ATCC 6962), *Salmonella enterica* Infantis (ATCC 51741) and *Salmonella enterica* Senftenberg (ATCC 8400), previously kept in Cryobeads at -80 ºC, were streaked onto tryptic soy agar (TSA, BD Difco, Sparks, MD). For the duration of the study, cultures were stored at refrigerated temperatures and transferred into fresh media periodically. *S*. Thompson, *S*. Newport, and *S*. Infantis were selected because frequently linked to pet food outbreaks (Crowe et al, 2005, Imanishi et al, 2014, Pitout et al, 2003). *S*. Senftenberg was selected based on its thermal tolerance and association with commonly rendered raw products (Kinley et al, 2010, Gong & Jiang, 2017). Low and high concentration *Salmonella* cocktails were prepared for the inoculation procedure as explained below with a final concentration of ~10^5 CFU/mL and ~10^7 CFU/mL, respectively.

Inoculation procedure.

One *Salmonella* colony from each TSA plate was transferred to 10 mL of tryptic soy broth (TSB, BD Difco, Sparks, MD) and incubated overnight at 37°C. Then, 500 µL of each solution was transferred into 50 mL of TSB and grown overnight. To prepare the low inoculum, 250 µL of each overnight culture was transferred into 25 mL of sterile 0.1 % peptone water (PW, BD Difco, Sparks, MD) and equal amounts of each strain was mixed in order to obtain a low inoculum of ~10^5 CFU/mL. For the high concentration preparation, overnight cultures were centrifuged for 10 min at 4000 rpm. Supernatants were then discarded, and pellets suspended in 25 mL 0.1% PW. Pellets were completely dissolved by vortexing, and equal amounts of each strain were mixed in order to obtain a total concentration of ~10^7 CFU/mL. Concentrations were confirmed by serial dilution on TSA (BD Difco, Sparks, MD). Fat samples were warmed to approximately 35 °C to

increase fluidity using a bucket heater (WG05 Insulated pail band heater, WarmGuard, Salt Lake City, UT). Either low or high inoculum was the added to fat samples at a liquid-to-fat ratio of 25 mL per 450 g of fat. For each type of fat (white grease and beef tallow), the inoculated sample was then divided in four different beakers of ~ 100 g. Four different moisture levels were obtained by adding deionized (DI) water at final volume of 0.0, 0.5, 1 and 3.0 %. The amount (mL) of DI water to add was calculated based on the mass balance equation (Lang & Steinberg, 1980). Samples were stored for 7 days at 48 or 76 ºC and *Salmonella* populations was evaluated daily. Chicken fat samples without any added water were used for comparison purposes.

Salmonella enumeration.

At each sampling time, 10 mL of the sample was pre-enriched in 90 mL of Brain Heart Infusion Agar (BHI, BD Difco, Sparks, MD) at 35 ± 2°C for 24 ± 2 h and then enriched in Rappaport-Vassiliadis (RV, BD Difco, Sparks, MD) broth at 42°C for 24 h. Samples were then serial diluted and plated on Xylose Lysine Deoxycholaye Agar (XLD, BD Difco, Sparks, MD). Typical *Salmonella* black colonies were counted to report on the remaining *Salmonella* population.

Survival kinetics determination.

OriginPro Lab Software (version 8) was used to determine the parameters of Weibull model (Equation 1) based on our earlier work (Trinetta et al., 2019) where the parameters α and β represent a characteristic time and curve shape, respectively.

Equation 2.1. Survival Kinetics Determination

$$logS(t) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta}$$

The goodness of the fit of the model was assessed using regression coefficients and least square errors. This model was applied only for samples challenged at 48 ºC, since at 76 ºC the count are below detectable limits after 24 hours.

Statistical analysis.

Each experiment was conducted in triplicate. All data collected were converted in log CFU/ml. Means and standard deviations were compared using Excel (Microsoft Corp., Redmond, WA). Statistical differences were evaluated using SAS® software.

2.5 Results and Discussion

Table 2.1 shows lipid, moisture percentage, and water activity measurements for rendered fat samples selected in this study. Rendered animal fatty acid composition is instead reported in Table 2.2. In all fat types, the major fatty acid was oleic acid followed by palmitic acid and linoleic acid. Although there was no significant difference *(P* > 0.05) in total saturated fat composition, the major saturated fatty acid (palmitic acid) was significantly higher (%) in chicken fat, as compared to choice white grease (Table 2.2) ($P < 0.05$). A significant difference ($P < 0.05$) was also observed in mono- and poly-unsaturated fatty acid fractions for the different fat sample types, with oleic acid greater in choice white grease as compared to chicken fat. As expected, in addition to the major fatty acids of animal fats, low to moderate amounts of $C_{18:3}$ and $C_{20:4}$ fatty acids were reported in our samples, and they were generally reflective of what others have reported for beef tallow, choice white grease, and poultry fat (Alm, 2013). Beef tallow was found to have a significantly higher percentage of arachidonic acid (20:4) compared to choice white grease and

chicken fat ($P < 0.05$). Choice white grease was found to have a statistically higher percentage of myristic (14:0), stearic (18:0), oleic acid (18:1), eicosenoic acid (20:1) and eicosadienoic acid (20:2), while low percentage of palmitoleic (16:1) and alpha-linoleic acid (18:3:3) were observed ($P < 0.05$). Significantly higher percentages of palmitic acid (16:0) and linoleic acid (18:2) were instead observed in chicken fat ($P < 0.05$).

A significant difference ($P < 0.05$) was observed in water activity, with choice white grease having higher values as compared to beef tallow and chicken fat (Table 2.1). These differences are probably attributed to variations in residual moisture introduced during handling and transportation of various rendered fats, rather than differences in animal species origin.

Subsequently, fat samples were incubated at different temperatures and moisture levels. Wet inoculation was used to mimic the introduction of moisture that could take place during transportation and storage. No variations in *Salmonella* concentrations were observed between moisture levels and/or inoculum type at 76 ˚C (data not reported). All *Salmonella* concentrations were below detectable limits after 24 hours (1 CFU/ml). These results were reported for each different type of animal fat analyzed in this study. Only, temperature was identified as significant factor on *Salmonell*a inactivation ($P < 0.05$). Similar results were also reported in our previous study when chicken fat samples were stored at 76 ˚C (Trinetta et al., 2019).

Figure 2.1 reports the data obtained when choice white grease and beef tallow were challenged with a high or low level of *Salmonella* wet inoculum and incubated at 48 ˚C. After 3 days, *Salmonella* populations were below detectable limit in all the samples ($P < 0.05$) (Figure 2.1 and. 2.2). The control samples (no addition of water) followed a similar trend. When a low wet inoculum was used, a progressive decline in *Salmonella* population was observed in all samples within 4 days, indicating extended survival (Figure 2.1B). No statistical differences were observed

between moisture levels ($P > 0.05$). No variations in *Salmonella* concentrations were observed between moisture and/or inoculum levels since all counts were below detectable limits after 48 hours also for beef tallow (Figure 2.2).

The Weibull model was fit to mechanistically explain the effect of water content on *Salmonella* survival kinetics for samples challenged with high or low concentrations of *Salmonella*. The model parameters β (shape parameter related to heat resistance) and α (hazard rate or scale parameter) are given in Table 2.4. When the heat resistance of cells increases, the survival kinetics show a concave upward shape ($\beta < 1$), while a concave downward survival curve ($\beta > 1$) indicates the heat resistance of cells decreases with heating time (van Boekel, 2002). The strong correlation between the model parameters α and β is also a good indication of the reliability of the analysis and performance of the model. Larger death rate kinetic values were found in the previous poultry fat study, compared to death rates found for beef tallow and white grease in this study (Trinetta et al, 2019). The Weibull model indicated that the inactivation kinetics show first order kinetic behavior. A low temperature inactivation is dependent by time, confirmed by a large α parameter (a characteristic time): at concentrations higher than 1% the survival kinetics approaches to first order kinetics (i.e., heat resistance does not depend on time) with a decreasing hazard rate.

Previous data collected by our group suggested that moisture percentage largely did not affect *Salmonella* growth in rendered fats (Trinetta et al, 2019). Water activity (a_w) is the scale of measurement (0.1-1.0) for the ratio of water vapor pressure in a food product compared to distilled water, bacteria generally require 0.86 a_w and above (Jay, Loessner & Golden, 2005). Many studies have been conducted to determine factors that influence *Salmonella* survival in low a_w foods (below 0.7). Farakos, Schaffner and Frank (2014) concluded that the major parameters affecting

Salmonella survival are: 1) food composition, 2) water activity and 3) temperature. According to their study, *Salmonella* exhibited increasing persistence with decreased water activity due to the presence of fat protected bacteria cells from inactivation (Farakos, Schaffner & Frank, 2014). The protective effect of fat was also observed by Li et al. (2014), who hypothesized that *Salmonella* may persist in microenvironments within high protein and high fat food mixes.

Few studies have been conducted on the effect of fatty acid composition and pathogen growth. Zhang et al. (2016) measured the antimicrobial effect of sophorolipids with and without the addition of a palmitic, steric or oleic acid base (2%) on *Salmonella* and *Listeria* spp. populations. It was observed that the inclusion of palmitic, steric and oleic acid resulted in no significant difference in antimicrobial activity of sophorolipids (Zhang et al., 2016). This observation might be valid also in our study, indicating that the differences observed between beef tallow, white grease and chicken fat levels of palmitic, steric and oleic fatty acids likely do not have an effect on *Salmonella* survivability. The significant and rapid reduction in *Salmonella* population was likely a function of increased temperature. Regardless of moisture level, inoculum level, or contamination level, holding fat at 76°C resulted in minimal detectable *Salmonella* after 24 hours. This is in agreement with other research which measured bacteria presence in rendered fat products at various temperatures and reported reductions in bacteria to undetectable levels in less than 1 hour (Trinetta et al, 2019; Ramirez-Hernandez et al. 2018). Conversely, moisture percentages had an effect in *Salmonella* survival in rendered fat when samples were held at 48ºC, but no differences among moisture % were observed.

The data collected in our research suggests that residual moisture in containers during transportation of white grease and beef tallow fat largely does not impact *Salmonella* growth. If contaminated with a high level of *Salmonella* (10^8 cfu/mL) and held at a low temperature (48°C),

moisture may influence the thermal death due to differences in water activity and water mobility kinetics.

2.6 Conclusions

The present research suggests that residual moisture in containers during transportation of white grease and beef tallow fat largely does not impact *Salmonella* spp. If contaminated with high levels of *Salmonella* (10^8 cfu/mL) and held at a low temperature (48°C), the pathogen may persist for several days and have the potential to contaminate end products.

2.7 Acknowledgments

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Table 2.1.

Chemical and Physical Characteristics of Rendered Animal Fats Selected for This Study Prior to Start Experiments

	Beef Tallow	**Choice White Grease**	**Chicken Fat**
Lipid %	81.9 + 1.55 [a]	79.2 + 1.98 [a]	82.8 + 1.12 [a]
Moisture %	0.11% + 0.03 [a]	0.16% + 0.00 [b]	0.06% + 0.02 [c]
Water Activity a_w	0.34 + 0.01 [a]	0.44 + 0.01 [b]	0.37 + 0.03 [a]

Results are presented as average values (of three replicates) and ± indicates standard deviation.

Different letters indicate significant difference (P<0.05) by row.

Table 2.2.

Major Fatty Acid Composition (Percent of Total Fatty Acids by Weight) of Rendered Animal Fats

	Beef Tallow	**Choice White Grease**	**Chicken Fat**
Myristic acid (14:0)	0.58 ± 0.10 [a]	1.63 ± 0.09 [b]	0.63 ± 0.05 [a]
Palmitic acid (16:0)	25.6 ± 0.63 [a]	23.5± 0.10 [b]	27.2 ± 0.36 [c]
Palmitoleic acid (16:1)	6.64 ± 0.26 [a]	2.84 ± 0.03 [b]	6.58 ± 0.05 [a]
Stearic acid (18:0)	6.35 ± 0.17 [a]	8.76 ± 0.20 [b]	6.14 ± 0.09 [a]
Oleic acid (18:1)	38.6 ± 0.70 [a]	43.8 ± 0.05 [b]	36.8 ± 0.26 [c]
Linoleic acid (18:2)	20.3 ± 0.28 [a]	17.0 ± 0.04 [b]	21.1 ± 0.18 [c]
Alpha-linoleic acid (18:3:3)	0.97 ± 0.07 [a]	0.62 ± 0.01 [b]	0.86 ± 0.05 [a]
Eicosenoic acid (20:1)	0.25 ± 0.05 [a]	0.82 ± 0.02 [b]	0.25 ± 0.01 [a]
Eicosadienoic acid (20:2)	0.11 ± 0.00 [a]	0.69 ± 0.01 [b]	0.11 ± 0.01 [a]
Arachidonic acid (20:4)	0.69 ± 0.08 [a]	0.43 ± 0.00 [b]	0.31 ± 0.02 [b]

Results are presented as average values (of three replicates) and ± indicates standard deviation.

Different letters (a, b, c) indicate significant difference (P<0.05) by row.

Table 2.3

Water activity values when fat samples were stored at 76ºC at 0 hour and 48 hours.

	Beef Tallow	Beef Tallow	Beef Tallow	Beef Tallow	Choice White Grease	Choice White Grease	Choice White Grease	Choice White Grease	Chicken Fat
	0%	0.5%	1.0%	3.0%	0%	0.5%	1.0%	3.0%	0%
0 hour	0.26 ± 0.07	0.44 ± 0.18	0.51 ± 0.24	0.63 ± 0.19	0.38 ± 0.09	0.63 ± 0.20	0.64 ± 0.11	0.87 ± 0.03	0.57 ± 0.18
48 hours	0.19 ± 0.03	0.20 ± 0.04	0.19 ± 0.02	0.45 ± 0.23	0.19 ± 0.05	0.19 ± 0.03	0.18 ± 0.02	0.28 ± 0.12	0.20 ± 0.05

Table 2.4

Selected Salmonella *cocktail survival kinetics in white grease and beef tallow challenged at 48 °C with different moisture levels.*

Inoculum condition	Fat type	Moisture level	Kinetics parameters	
			α (day)	β
High	White grease	0.0%	0.47±0.07	1.91±0.18
		0.5%	0.37±0.14	1.62±0.35
		1.0%	0.24±0.03	1.13±0.07
		3.0%	0.18±0.07	1.12±0.16
	Beef tallow	0.0%	0.84±0.23	3.30±0.91
		0.5%	0.89±0.06	3.41±0.29
		1.0%	0.87±0.04	3.26±0.12
		3.0%	0.59±0.17	2.27±0.56
Low	White grease	0%	1.96±0.07	5.08±0.28
		0.5%	0.50±0.14	1.17±0.17
		1.0%	0.52±0.25	1.17±0.27
		3.0%	0.98±0.63	1.90±0.95
	Beef tallow	0.0%	0.76±0.28	2.62±0.14
		0.5%	0.56±0.14	2.51±0398
		1.0%	0.90±0.39	2.65±0.25
		3.0%	0.87±0.36	2.70±0.21

Figure 2.1

Salmonella *cocktail remaining population in white grease samples challenged at 48°C with different wet inocula levels (high (A) and low (B)), and moisture level (0%, 0.50%, 1% and 3%) over time*

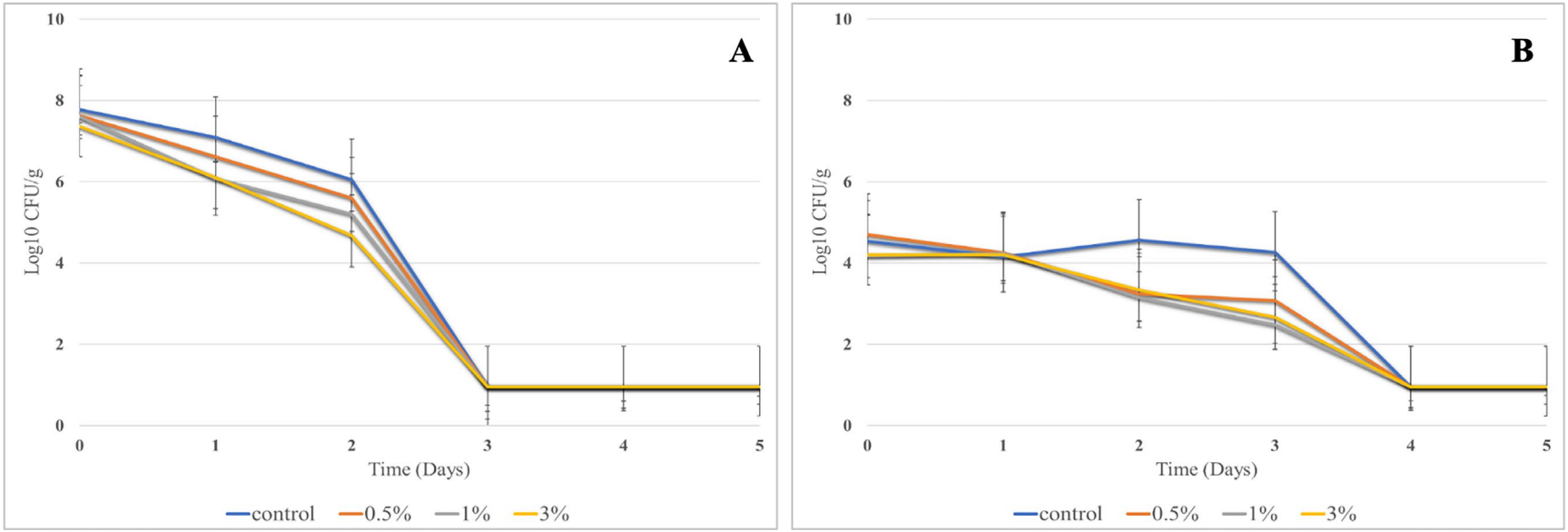
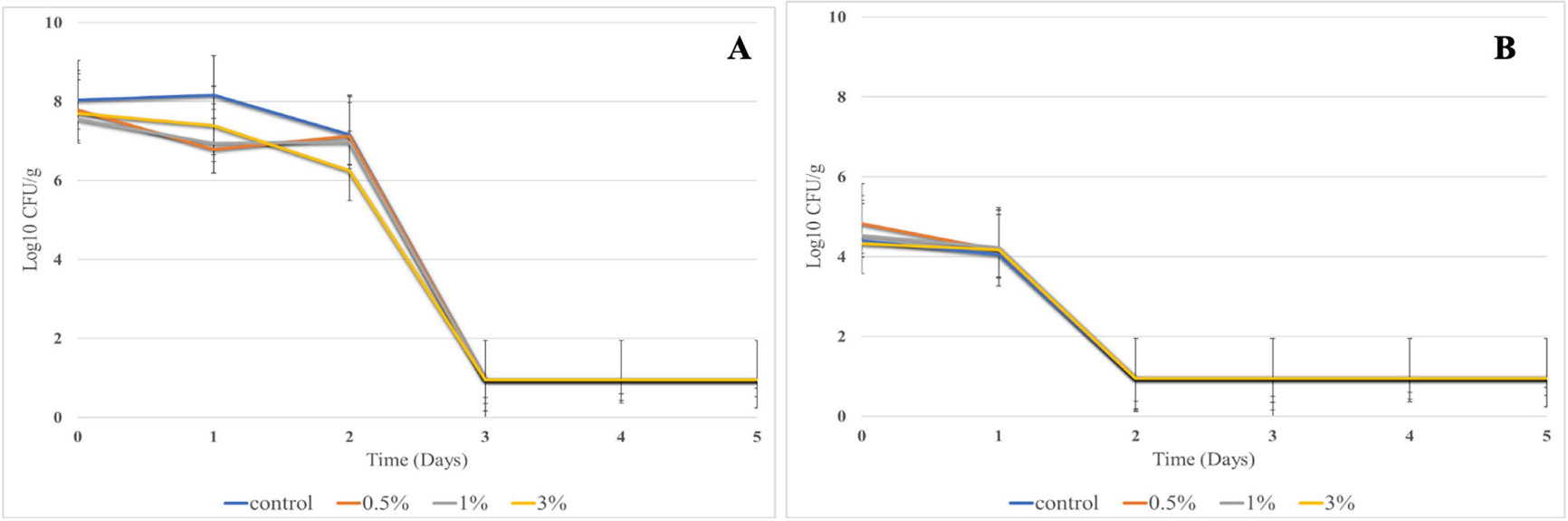


Figure 2.2

Salmonella *cocktail remaining population in beef tallow samples challenged at 48°C with different wet inocula levels (high (A) and low (B)), and moisture level (0%, 0.50%, 1% and 3%) over time.*



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Chapter 3 - Quantitative and Qualitative Assessments of *Enterobacteriaceae*, Coliforms and Generic *Escherichia coli* on Fresh Vegetables Sold in Cambodian Fresh Produce Distribution Centers

3.1 Abstract

Cambodia has introduced several initiatives to increase production and consumption of fresh produce throughout the country. Fresh produce, however, is often associated with foodborne disease; thus, understanding how foodborne pathogens enter and are transmitted throughout Cambodian production chains can help to ensure positive nutritional outcomes from increased produce consumption. Toward this end, this study was conducted to provide a quantitative and qualitative assessment *Enterobacteriaceae*, coliforms and *Escherichia coli* on tomatoes, cucumber, and lettuce sold through a produce distribution center in Cambodia. Samples (n = 384) were collected over six-month period (December 2019 - May 2020) and screened for the presence of *Enterobacteriaceae*, coliforms and *E. coli* following methods adapted from the Food and Drug Administration's Bacteriological Analytical Manual. A significantly greater concentration of *Enterobacteriaceae* and coliforms were observed on lettuce, compared to cucumber and tomato level ($P < 0.05$). The indicator organisms were present at the highest frequency ($P < 0.05$) in lettuce, followed by cucumber and tomato. The results of this study provide an initial assessment *Enterobacteriaceae*, coliforms and *E. coli* contamination in vegetables sold through Cambodian markets. These data can be used to identify sources of contamination and to develop interventions which limit contamination and ensure the safety of fresh produce available to Cambodians.

3.2 Introduction

Sanitation is described as the process of creating and promoting hygienic and healthful conditions and the practices that help maintain such environments (Marriott, Schilling, and Gravani, 2018b). Measuring the presence and concentration of microorganisms from farm to fork can inform the allocation of attention and resources to reduce food safety risk. The family of *Enterobacteriaceae* is composed of 10 genera and 20 clinically significant species (Rock and Donnenberg 2014). These microorganisms are also considered indicators for food quality and hygiene (Cordier, 2006, Anand & Griffiths, 2011, Halkman & Halkman, 2014). In general, the presence of *Enterobacteriaceae* may indicate poor sanitation, hygiene and handling practices (Marriott et al., 2018a; 2018b).

Coliforms are part of the family of *Enterobacteriaceae*, and include species such as *Citrobacter*, *Enterobacter* and *Escherichia coli* (Halkman & Halkman, 2014). This subgroup makes up about 10% of intestinal microflora in humans and animals and it their presence on food is often used as indication of possible fecal contamination (Cordier, 2006, Anand & Griffiths, 2011). Likewise, as many foodborne pathogens are enteric organisms, the presence of fecal contamination in food products may lead to exposure of harmful bacteria such as pathogenic *E. coli* (Rock and Donnenberg 2014). This harmful microorganism is responsible for the majority of diarrheal diseases in developing countries, such as Africa and Southeast Asia (Havelaar et al. 2015).

Approximately one-third of diarrheal-related illness in developing counties are associated with the consumption of contaminated food (Havelaar et al. 2015, Mokomane et al. 2018, Ugboko et al. 2020). For many of these countries, however, few studies exist that clearly identify specific etiological agents (e.g., pathogenic *E. coli*, *Salmonella*) responsible for foodborne disease

outbreaks (Roesel & Grace, 2014). Nevertheless, fresh produce and other horticulture products have been linked to foodborne pathogens and outbreaks worldwide (Olaimat & Holley, 2012; Franz & van Bruggen, 2008; Roesel & Grace, 2014) and Cambodian diets, like many of those in South East Asia, contain many raw fruits and vegetables. (Murshid 1998). As in other countries in the Greater Mekong Region Subregion, qualitative assessments of food contamination rates in Cambodia are still limited and scarce. Understanding how microorganisms are transmitted along the food production chain is likely key to develop intervention strategies for food safety.

Previous studies (Desiree et al., 2020b) have provided initial characterizations of contamination along vegetable production chains due to deficiencies in food safety practices in informal produce markets in Cambodia. Informal markets represent the main point for retail purchasing a variety of food products, including vegetables, fruits, meat and household items in Cambodia. Such markets have been described as locations that "escape effective health and safety regulations, and are often untaxed and unlicensed" (Roesel and Grace 2014; Sokhen, Kanika, and Moustier 2004).

Before reaching retail informal markets, many food products (e.g. produce) in Cambodia are gathered and collected in distribution centers before being redistributed to domestic retailers and/or international wholesalers. The objective of the study presented here was to better understand the role of the distribution center in farm-to-fork food safety continuum by measuring indicator microorganisms that reflect sanitation and hygiene practices in a major Cambodian fresh produce distribution center.

3.3 Materials and Methods

Produce distribution center.

Produce distribution centers in Cambodia are a transition point for bulk products such as produce, meat, nuts, as well as non-food products. The samples described below were collected from a distribution center in Battambang Province (Figure 1), located approximately 291 km northwest of Phnom Penh. The Battambang distribution center is the only distribution center in the northwestern region and therefore represents one of the main centralized centers in the Cambodia vegetable value chain.

Sample collection.

Three vegetable types (tomato, cucumber, lettuce) were purchased from the vendors in the distribution center. A total of 384 samples were collected during this study: tomato (n=145), cucumber (n=132) and lettuce (n=107). Samples were collected over six months (December 2019 – May 2020). Immediately after collection, vegetables were aseptically transferred into separate, sealable bags, labeled, and stored in ice. 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 for microbial analysis within 24 hours of collection.

Sample preparation.

Produce samples were prepared for microbiology analysis by aseptically transferring 10 ± 0.2 g of sample into individual filtered sterile bags. Buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD, USA) was added to the bag (90 mL) and samples were hand stomached for 2 min. Samples were serially diluted in 0.1% peptone water (Becton,

Dickinson and Company, Sparks, MD, USA) and plated as to determine viable cell counts as described below.

Quantification of Enterobacteriaceae, coliforms and generic *E. coli*.

Diluted samples were transferred to *Enterobacteriaceae* Petrifilm and *E. coli*/Coliform Petrifilm (3MTM, Minneapolis, MN-USA) and incubated at 35 ± 2°C for up to 48 hr. Red colonies with yellow zones and/or gas bubbles on *Enterobacteriaceae* Petrifilm plates were identified as *Enterobacteriaceae*. Red and blue colonies with gas on *E. coli*/Coliform Petrifilm plates were identified as coliforms while blue colonies with gas were identified as generic *E. coli*.

Statistical analysis.

Samples were plated in duplicate and average $\log_{10}$ CFU/g for each sample was calculated and used in data analysis. Means and standard deviations were obtained using Excel (Microsoft Corp., Redmond, WA). Statistical differences were evaluated using SAS® software PROC GLIMMEX (SAS Institute, Cary, NC, USA). A completely randomized design was used to compare values across vegetables (tomato, cucumber, lettuce). Differences were considered statistically significant at $P < 0.05$.

3.4 Results

Presence of typical colonies of *Enterobacteriaceae*, coliforms, or generic *E. coli* were considered to be positive using *Enterobacteriaceae* or *E. coli*/Coliform Petrifilm (3M^{TM}, Minneapolis, MN-USA) and used to calculate prevalence (%). A significantly higher level ($P <$ 0.05) of *Enterobacteriaceae* and coliforms were observed on lettuce, compared to cucumber and

tomato (Figure 3.2A; $P < 0.05$). Overall, the highest ($P < 0.05$) concentration of *Enterobacteriaceae* were observed on lettuce (4.71 ± 1.02 $\log_{10}$ CFU/g) followed by cucumber (3.44 ± 1.12 $\log_{10}$ CFU/g), and tomatoes (2.79 ± 1.02 $\log_{10}$ CFU/g). Comparable trends were observed for coliform concentration (Figure 3.2B) where lettuce had the highest concentrations of coliforms (4.36 ± 1.23 $\log_{10}$ CFU/g), followed by cucumber (3.28 ± 1.32 $\log_{10}$ CFU/g) and tomato (2.69 ± 1.45 $\log_{10}$ CFU/g). Vegetable type did not have a significant effect ($P > 0.05$) on concentrations of *E. coli* (Figure 3.2C; lettuce: 2.71 ± 0.97 $\log_{10}$ CFU/g; cucumber: 2.28 ± 0.95 $\log_{10}$ CFU/g; tomatoes: 2.18 ± 0.16 $\log_{10}$ CFU/g).

Overall, the highest ($P < 0.05$) prevalence of *Enterobacteriaceae* was observed in lettuce samples (98.1% ± 5.44) followed by cucumber (86.6% ± 13.08) and tomato (65.8% ± 12.64) samples. A similar trend was observed for coliforms, with the prevalence of coliforms highest in lettuce (96.8% ± 8.16; $P < 0.05$), when compared to both cucumber (84.9% ± 10.86) and tomato (62.6% ± 15.44). Generic *E. coli* was observed at a lower prevalence across samples with the highest prevalence of *E. coli* found in lettuce samples lettuce samples (28.9% ± 12.64; $P < 0.05$), followed by cucumber (9.0% ± 6.97) and tomato (1.2% ± 1.88) samples.

3.5 Discussion

The concentrations of *Enterobacteriaceae*, coliforms, and *E. coli* in Cambodian vegetables described here indicate that effective sanitary practices are not likely to be in place at produce distribution centers in Cambodia. As shown in figure 3.1, several transportation vehicles were allowed in the market and produce was accumulated and stored directly on floors, increasing the risk for bacterial and pest contamination. Furthermore, as seen in similar informal markets, vegetables were frequently stored and sold at ambient temperature and directly exposed to the

environment without any protection against insects and rodents (Nidaullah et al. 2017; Schwan et al. 2020).

Levels of indicator microorganisms were higher on lettuces, followed by cucumber and tomatoes. Produce characteristics, such as composition, surface morphology, pH and moisture availability, as well as differences in growing conditions, transportation and storage procedures, play a significant role in bacterial contamination and growth (Leff and Fierer, 2013). Similarly, Desiree et al., (2020b) found higher levels of coliforms and *E. coli* on lettuce, as compared to tomatoes and cucumber collected from informal markets in Cambodia. The vegetable value chain, as previously described by Desiree et al. (2020a), is complex and affected by several factors at different points. For example, at the distribution center level, food safety challenges include lack of hygiene and sanitation practices, food safety regulations, and infrastructure, as well as the absence of clean water and proper storage conditions (Schwan et al. 2020; Desiree et al. 2020a). Strategies to improve food safety interventions at the distribution level might effectively control the burden of microbial disease. For example, the use of disposable gloves when handling produce, washing hands frequently, especially after handling money or using the restroom, keeping produce off the ground and cleaning food-contact surfaces frequently could help control microbial contamination at the distribution level and improve safety of vegetables available to Cambodians.

3.6 Conclusions

Results from this study provide an initial assessment of *Enterobacteriaceae*, coliform and generic *E. coli* carriage rates in Cambodian vegetables at distribution centers and highlight the need to establish and/or improve sanitation interventions within and in-between the different points of the food supply chain. Future research should evaluate the presence and concentrations of

specific foodborne pathogens in the Cambodian vegetable value chain and identify intervention strategies that could be most effective within the context of Cambodia informal vegetable markets.

3.7 Acknowledgment

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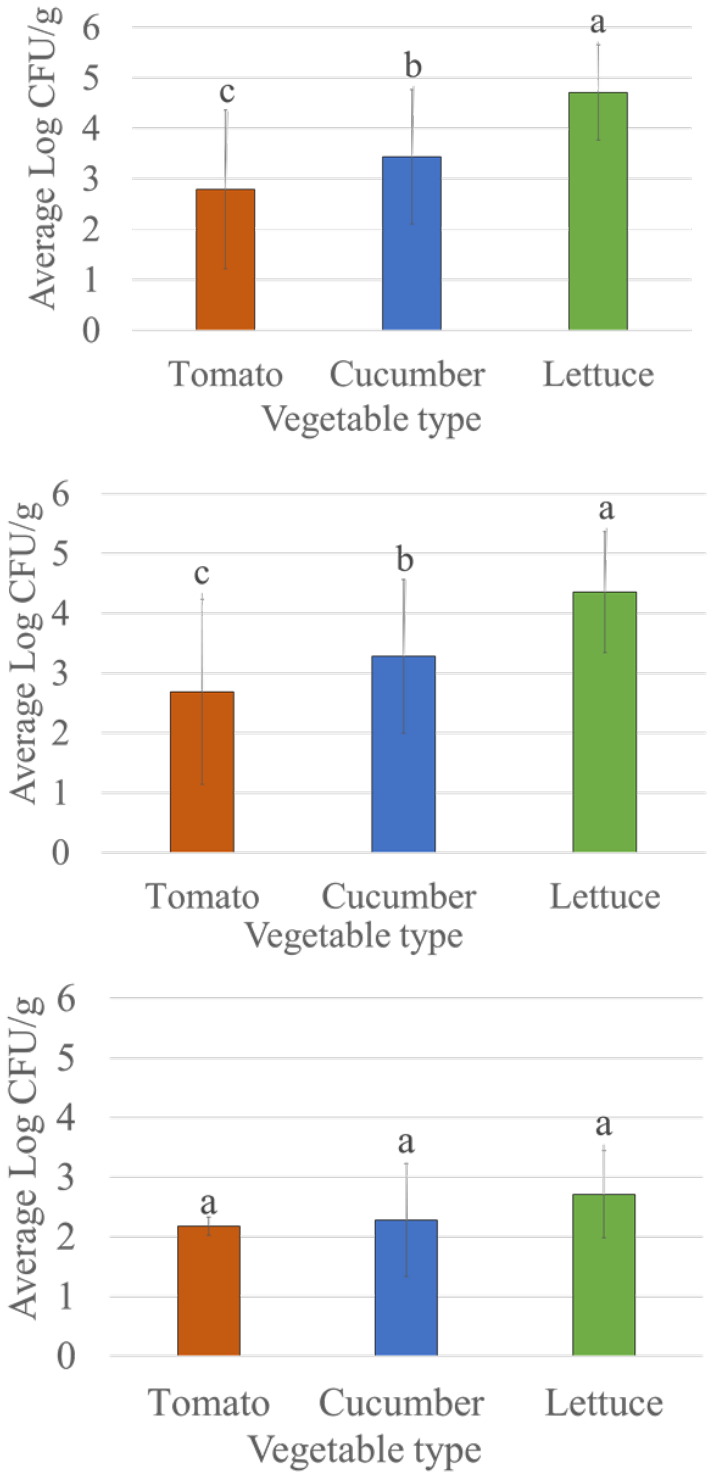
Figure 3.1.

Vegetable Distribution Center at Battambang Province in Cambodia

Figure 3.2

Concentrations of Enterobacteriaceae *(A), coliform (B), and generic* E. coli *(C) on tomato, cucumber and lettuce expressed as average* log_{10} *CFU/g.*



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Chapter 4 - Conclusions

The studies presented in this document provide insight into the importance of food safety controls to improve public health outcomes. These studies represented two major food commodities in two countries: rendered fat (US) and vegetables (Cambodia). Different food matrixes and the effectiveness of food safety infrastructure within a country dictate the most important food safety controls to implement. Conducting research which adds to the current level of food safety knowledge for a commodity in a particular environment is key for selecting the most effective food safety interventions, leading to the most successful public health outcomes.

The study in chapter 2 evaluated process control parameters (i.e., storage temperature, moisture percentage, and holding time) and their effect on *Salmonella* survivability in rendered fat. The findings from this study expand on current rendered product food safety. Previously, rendered products were thought to be free of potentially pathogenic organisms post-processing. However, outbreaks involving dry pet food and animal feed highlight a research opportunity to better understand conditions in which rendered products may be a source of contamination. A potential food safety process control identified by this study is increased temperatures during transportation and storage (76ºC). These preventative measures have the potential to positively impact public health through enhanced food safety of pet food and animal feed. Enhanced pet and animal feed safety has both a direct and indirect effect on public health. Contaminated pet and animal feed may cause human illness directly, such as handling and accidental consumption of contaminated feed, or indirectly, such as an animal shedding pathogenic bacterium in home environments. Making adjustments to rendered product handling, storage and transportation to increase food safety will reduce the risk of human and animal FBD.

In our study, three rendered fats were measured for several physical and chemical characteristics: i) lipid percentage (%), ii) moisture percentage (%), iii) water activity (a_w), and iv) fatty acid composition. No significant connection was found between a physical or chemical fat characteristic and an effect on *Salmonella* survivability. Despite this, the choice white grease, beef tallow and chicken fat characteristic data may be used in future studies to validate fat composition or make a new discovery of composition effect. Conducting environmental testing throughout finished rendered products transit route is a logical next step for research into rendered product safety. Such a microbial survey could identify potential contamination sources for rendered finished products or conditions which increase the hazard risk. Additionally, further studies may consider conducting rendered fat food safety experiments on a larger scale (volume of fat) with industry indicative equipment.

The study in chapter 3 utilized a quantitative and qualitative assessment of indicator organisms (*Enterobacteriaceae*, coliforms and generic *E. coli*) to understand the baseline hygiene levels of vegetables sold through a vegetable distribution center in Battambang, Cambodia. This study, alongside similar microbial surveys, can be used to draw conclusions on the most effective locations for food safety interventions. Examples of food safety interventions that may be effective at strategic locations include: i) hand washing stations; ii) cleaning of food contact surfaces; iii) improved sanitary transportation practices; and iv) increased food safety oversight. In chapter 3, lettuce was found to have the highest level ($\log_{10}$ CFU/g; *Enterobacteriaceae* and coliform) and prevalence (%; *Enterobacteriaceae*, coliform and generic *E. coli*) of indicator organisms. Possible explanations for these results include the growing conditions (i.e., on the ground or on a vine) or the surface morphology of lettuce (compared to cucumber and tomato). The results from this study

will help to inform the strategic placement of food safety process controls, thus, reducing the risk of FBD for Cambodians who purchase their vegetables from the informal value chain.

Finally, our study did not examine the relationship between vegetable contamination and the season change in Cambodia. Actors in the vegetable value chain in Cambodia experience two main seasons: 1) dry season; and 2) wet season. Season may play a role in microbial dynamics across the vegetable value chain due to environmental differences, such as rain water, increased contact with contaminated water sources, and damp food contact surfaces. Thus, seasonal differences may dictate varying needs for sanitation process controls to maximize public health outcomes. Overall, the results from this study, along with other sources, will be valuable in informing strategic food safety focused process controls. Furthermore, future studies at different points along the Cambodia informal vegetable value chain are needed. More data describing the microbial dynamics of indicator organisms will inform the sanitation needs of the Cambodia informal vegetable value chain. Moreover, more advanced laboratory analysis to discover the predominant pathogenic bacteria would provide a better image of the public health impact of the informal vegetable food chain.

The findings in chapter 2 and 3 shed light on food safety knowledge gaps for U.S. rendered product handling and the informal vegetable value chain in the northeastern region of Cambodia. To ensure a safe animal food product, finished rendered products transportation and storage conditions should be monitored. The findings in chapter 2 revealed *Salmonella* contamination, if present due to high level of cross contamination, may persist in rendered white grease and beef tallow for up to four days at lower storage temperatures (48ºC). The findings in chapter 3 provided a baseline understanding of contamination and lack of sanitation at a distribution center along the informal Cambodian vegetable value chain to eventually enhance food safety from farm to fork.

Combined, these studies shed light on the importance of monitoring food safety, regardless of food commodity or country food safety status.