POST-HARVEST TRANSMISSION OF *Salmonella enterica* TO THE ROOTS AND LEAVES OF INTACT PACKAGED BUTTERHEAD LETTUCE

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ABSTRACT

In the United States, illnesses associated with fresh produce are increasing in frequency. While contamination risks are present at every aspect of the farm to fork continuum, post-harvest practices holds the potential for cross-contamination of large amounts of product. Post-harvest contamination risks for hydroponically grown lettuce packaged with intact roots and sold as 'living lettuce' are poorly understood. In this study, transmission of *Salmonella enterica* serotype Enteritidis to the roots and leaves of butterhead lettuce was studied when contamination was introduced during typical handling practices. The effectiveness of random sampling strategies for selection of *Salmonella* contaminated leaves was assessed by co-inoculating the *Salmonella* solution with Glo Germ™ and comparing recovery from blacklight selected leaves. The recovery of *Salmonella* was improved by only 0.5 log CFU/g when blacklight was used to select Glo Germ™ contaminated leaves (P=0.05). This suggests random leaf selection as described by current FDA protocols is adequate. In addition, this study showed rapid transfer of *Salmonella* from liquid to the roots and sub-sequentially to the leaves of living lettuce. *Salmonella* persisted but did not grow on leaves when stored at 4˚C for 18-days. Storage at 12˚C was associated with 2 log CFU/g increases in *Salmonella* on roots after 18-days storage (P=0.0002), while 4˚C storage was associated with a decrease of 0.4 log CFU/g *Salmonella* on roots (P=0.0001). Growth occurred only under temperature abuse conditions. This reinforces the need for maintaining temperature control and highlights the importance of identifying risks associated with post-harvest handling during hydroponic production and distribution.

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DEDICATION

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ATTRIBUTION

Several people contributed to the completion of the research described in this thesis, and a description of their contributions are included below.

Monica A. Ponder- Ph.D. (Department of Food Science and Technology, Virginia Tech) is the primary advisor and head of the committee chair. Dr. Ponder provided positive guidance in the development of this research project, and resources to fund this study. Her background knowledge in food safety and food microbiology significantly contributed to this project.

David D. Kuhn- Ph.D. (Department of Food Science and Technology, Virginia Tech) was an active member of the committee and provided expertise and support in hydroponic system set-up at the Southwest Virginia Aquaculture Research Center , Virginia. His mentorship and knowledge greatly contributed to this research project.

Gregory E. Welbaum-Ph.D. (Department of Horticulture, Virginia Tech) was an active member of the committee and provided expertise in horticultural crops and assistance in the living lettuce production. His mentorship and knowledge greatly contributed to this research work.

Daniel Taylor- (Department of Food Science and Technology, Virginia Tech) provided background knowledge in designing hydroponic system and was responsible for growing the butterhead lettuce used in this project at the Southwest Virginia Aquaculture Research Center in Saltville, Virginia.

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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

For thousands of years, food safety has been a concern for consumers simply because food is recognized as a potential carrier for harmful bacteria. Food-borne illnesses present a serious public health burden worldwide. The overall burden of food-borne illness in the United States is estimated at 48 million cases per year and estimated costs of food-borne illnesses are $152 billion per year *(9)*. Ingestion of food contaminated by bacteria, parasites or viruses results in illnesses for one in six Americans each year *(3)*. Outbreaks, defined as two or more people with the same symptoms and identical etiological agent, are increasingly attributed to fresh produce. Outbreaks attributed to consumption of fresh produce have increased from <1% (13/1,857 outbreaks) in the 1970s to 6% (114/1,788 outbreaks) in the 1990s *(10)*. The frequency of produce outbreaks increased from two outbreaks per year in the 1970s to 16 per year in the 1990s *(10)*.

Food-borne outbreaks associated with leafy greens have increased by 38.6% *(6,7)*. "Leafy greens" refers to vegetables including lettuce, cabbage, endive, escarole, spinach, broccoli, collard greens, turnip greens, mustard greens and kale *(12)*. Leafy greens are recognized as a vehicle for transmission of pathogens that have the ability to cause food-borne illness outbreaks *(1, 2, 4, 6, 7)*. Between 1973-2006, a total of 502 food-borne outbreaks associated with leafy greens were reported in the United States, and 35 outbreaks were attributed to serotypes of *Salmonella enterica (6,7)*.

Pre-cut, pre-washed and packaged leafy greens that require little preparation are increasing in demand by consumers *(1)*. Since the 1960s, per capita consumption of different types of leafy greens have increased from 21.4 pounds per capita to a record high in 2004, with a

total lettuce consumption of 33.2 pounds per capita *(13)*. Lettuce is a leading food crop in the United States with an annual profit of more than 2 billion dollars in year *(14)*.

While the bulk of lettuce and leafy greens sold in the United States are field grown, hydroponic production in greenhouses is increasing in popularity. Much of the lettuce produced hydroponically is packaged with intact roots in a plastic clamshell and marketed as "Living Lettuce". The survival and behavior of human pathogens within hydroponic lettuce production systems is not well understood. It is known that post-harvest activities such as washing, handling, and packing can increase the amount of contaminated product through cross-contamination *(5, 6, 8, 9, 11)*. The objectives of this study were to (i) Quantify the transfer of *Salmonella enterica* serotype Enteritidis from inoculated roots to the leaves of mature Butterhead lettuce packaged as "living lettuce" in a clamshell with intact roots, (ii) Determine the survival of *S*. Enteritidis on roots and edible tissue of living lettuce stored at 4˚C and 12˚C throughout a typical product shelf life, (iii) To investigate the effectiveness of the current sampling strategies for quantification of *S*. Enteritidis on living lettuce. We hypothesize that post-harvest survival of *Salmonella enterica* from inoculated roots to the leaves can persist at FDA recommended storage temperatures and grow at temperature abusive conditions. We also hypothesize that sampling strategies can be improved to increase detection of *Salmonella* on leafy greens.

These objectives will provide important information about living lettuce and *Salmonella enterica* serotype Enteritidis during post-harvest handling. The techniques described here can be used to increase awareness of maintaining safe handling practices of living lettuce in hydroponic systems and guide risk management strategies. Therefore, understanding current harvesting, transport, and handling practices is important to identify potential risks and implement strategies to reduce cross-contamination. Control measures need to be identified to reduce risks and

improve the safety of leafy greens. These insights will be valuable in designing guidelines targeting post-harvest handling practices in commercial-scale, hydroponic production of leafy greens. Application of GAPs (Good Agricultural Practices) and GHPs (Good Handling Practices) are suggested to increase food safety of leafy greens by minimizing pathogen contamination during post-harvest handling.

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CHAPTER 2: LITERATURE REVIEW

Globalization of the food supply has made fruits and vegetables available regardless of season. Improvements in production yields, distribution, and trade policy allow perishable foods to travel long distances with minimal spoilage *(12)*. Consumers are more aware of the important nutrients and health benefits associated with eating fresh produce. Fruits and vegetables have fundamental nutrients that offer key dietary advantages such as essential minerals, vitamins, antioxidants and other substances that can reduce or prevent diseases with the ultimate goal of promoting a healthy lifestyle *(4)*. High quality produce subjected to minimal processing is in demand by consumers who are often willing to pay more for produce grown locally with sustainable methods.

Fresh produce-related illnesses monitored by Centers for Disease Control and Prevention surveillances (CDC) have been increasing from <1% in the 1970s to 6% in the 1990s and continues to rise *(73)*. The number of produce-related outbreaks increased from two outbreaks per year in the 1970s to 16 per year in the 1990s *(73)*. The sharp rise in produce-related outbreaks is an ever-increasing challenge for the food industry. A Produce Safety Project report from March 2010 estimated the annual economic burden of food-borne illness in the United States to be $152 billion, of which $39 billion was from economic losses associated with contaminated domestic produce *(68)*. CDC states that reducing food-borne disease by 10 percent would prevent 5 million people infected annually *(21)*. Widespread produce outbreaks have been documented including: *Salmonella* Braenderup infections from mangoes, *Escherichia coli* O157:H7 linked to spinach and romaine lettuce, *Salmonella* Newport associated with alfalfa sprouts, *Salmonella* Litchfield from cantaloupes, and *Salmonella* Typhimurium linked to tomatoes *(24)*.

Leafy greens are traditionally perceived as a relatively safe product, and it is difficult to convince consumers that such nutritious greens can be highly contaminated with pathogens. Leafy greens are recognized as a vehicle for transmission of pathogens that have the ability to cause food-borne illness outbreaks *(22, 23, 24, 25).*

Hydroponic leafy greens production has been increasing in popularity and the importance of contamination prevention must be emphasized at all stages of production. Prevention of contamination is vital to leafy greens safety and to maintain the economic value of the commodity. Further efforts are necessary to understand the preferred routes of contamination and the appropriate intervention to reduce the impact of food-borne outbreaks. Assessing continuing reports of food-borne disease outbreaks is critical towards the success of current and future prevention strategies. These insights will be valuable for developing safe hydroponic practices to improve pre- and post-harvest safety of leafy greens, particularly living lettuce.

Production Value, Per Capita Consumption, and Exported and Importation of Fresh Lettuce

According to the Economic Research Service (ERS) and United States Department of Agricultural (USDA) databases, the convenience of minimal processing such as pre-cut and pre-washed lettuce that requires little preparation is increasing in popularly *(81, 83, 85, 86).* It was reported that the per capita consumption of all lettuce varieties has been increasing since 1960 with 21.4 pounds per capita and in 2004 total lettuce consumption reached a record high of 33.2 pounds per capita *(85).* Since 2004, the per capita consumption has dropped slightly and in 2010 28.2 pounds per capita of lettuce was consumed *(85).* In terms of production value, lettuce is a leading vegetable crop in the United States with an annual profit of more than 2 billion dollars per year *(86).*

The majority of the US lettuce crop is consumed in country while some is also exported. The US exports lettuce to selected countries such as Mexico and Canada. In 2009, Mexico imported a net worth of $88,438,355 and 223,788,972 pounds while Canada imported a net worth of $25,106,690 and 66,609,395 pounds of all various lettuce *(77).*

Statistical Analysis of Food-borne Illnesses Outbreaks Burden in the United States

Food-borne illnesses present a serious public health burden in the United States. It is estimated that 48 million people will suffered from food-borne illness annually in the United States (*21*). Multiple recognized surveillance databases were utilized to estimate the overall food-borne illnesses burden in the United States. Data analysis was collected between 2000 and 2008 and based on US population in 2006 (299 million persons), food-borne illnesses were associated with one of the 31 known specific pathogens *(67).*

Table 2.1: The number of people affected by food-borne illnesses, hospitalizations, and deaths linked to 31 known pathogens in 2000-2008.

Overall estimate of 31 known pathogen contributors of Food-borne Diseases, 2000-2008 *(67)*.	
Food-borne illnesses	9.4,000,000
Hospitalizations	55,961
Deaths	1,351

The Food-borne Disease Outbreak Surveillance System survey concluded that between 2003-2007 the number of produce-related food-borne illnesses in the U.S. was 19,677,547 and that Virginia ranked number 12 in number of reported cases (500,395). California ranked number 1, contributing to 2,372,499 cases of produce-related food-borne illnesses *(68).* Norovirus and serotypes of non-typhoidal *Salmonella enterica* are the leading pathogens accountable for causing food-borne illnesses in the United States *(21).* Estimated annual number

of Norovirus illness was 5,461,731 in 2011 and non-typhoidal serotypes of *Salmonella* were 1,027,561 cases in 2011. Non-typhoidal serotypes of *Salmonella* and *Toxoplasma gondii* are food-borne pathogens that cause the largest number of deaths annually, with 378 and 327 cases, respectively *(21)*. In a 2011 journal, *Ranking the Risks: The 10 Pathogen-Food Combination with the Greatest Burden on Public Health*, poultry was rated first with a liability cost of $2,462,000 and 180 associated deaths recorded in the United States. Fresh produce was listed as fourth, accounting for a $1,405,000 cost of illness and 134 deaths *(9)*.

Produce-associated contamination between 1973 and 1997 accounted for 190 outbreaks, 16,058 illnesses, 598 hospitalizations, and 8 deaths *(73)*.

Table 2.2: The number of food-borne outbreaks, illnesses, and deaths cases associated with leafy greens reported from 1973-2006.

Food-borne Outbreaks Associated with Leafy Greens Reported From 1973-2006 *(67,68)*	
Outbreaks	502 (4.8%)
Illnesses	18,242 (6.5%)
Deaths	15 (4.0%)
Salmonella Outbreaks	35 (10.4%)

Notable Outbreaks Associated with Leafy Greens

Food-borne illness outbreaks can have a severe economic impact on the food industry. *Salmonella enterica*, a common food-borne disease, is a problem recognized internationally. In 2004, multiple illnesses have been linked with imported 'Rucola' lettuce (*Eruca sativa*-known as rocket salad or arugula in the US) and mixed salad blends in several European countries *(61)*. Three isolates of *Salmonella* Thompson were identified from arugula and confirmed as the outbreak strain by pulsed-field gel electrophoresis by the Norwegian Institute of Public Health

(NIPH) on November 15, 2004. The imported arugula was traced to an Italian producer and the contamination source was determined to be irrigation with non-potable water. This case emphasized the importance of using good water quality for leafy green production.

In the United States, contaminated minimally processed bagged spinach was linked to a multistate outbreak of food-borne, *Escherichia coli* O157:H7 *(22)*. The 199 people affected and 3 deaths were recorded. Of those infected, fifty-one percent were hospitalized and sixteen percent developed kidney failure due to hemolytic-uremic syndrome (HUS). The outbreak affected 26 states with Nebraska having the highest number of confirmed cases with 11. The FDA traced the outbreak to Natural Selection Foods LLC of San Juan Bautista, California and the spinach was likely contaminated by run-off from adjacent dairy pastures *(22)*.

A December 2011, multistate outbreak infected 60 people was linked to romaine lettuce contaminated by a strain of *E.coli* O157:H7 *(23)*. The outbreak was confirmed in 10 states: Arizona, Arkansas, Georgia, Illinois, Indiana, Kansas, Kentucky, Minnesota, Missouri, and Nebraska. There were 37 cases in Missouri the largest of any state. Amongst those who were infected, 30 were hospitalized and 2 were diagnosed with hemolytic uremic syndrome (HUS). An investigation was conducted to determine the original source of the outbreak in order to contain and prevent it from spreading. Evidence concluded that the romaine lettuce was served on salad bars at all locations of grocery store Chain A. The romaine lettuce came from a single lettuce processing facility distributor, which suggests that the romaine lettuce was contaminated during transportation prior to its arrival of the designated grocery store Chain A *(23)*.

Salmonella spp. outbreaks associated with lettuce have not been documented recently, however a number of recalls based on presence of pathogen have occurred, indicating there is still a potential risk of food-borne illness.

Table 2.3: Recalls reported from 2009-2012 associated *Salmonella enterica* with various salad blends from field production *(78).*

Brand name	Date of recall	Microorganism Identify	Product	Reason	Plan of Action	Location
Fresh Express	October 11, 2012	*Salmonella*	Hearts of Romaine Salad	A random sample revealed a positive result for *Salmonella*	Voluntary recall	North Carolina
Pacific International Marketing	July 6, 2012	*Salmonella*	Bulk Romaine Lettuce	*Salmonella* tested positive taken at the field production	Voluntary recall	California, Nevada
Dole Fresh Vegetables	April 14, 2012	*Salmonella*	Seven Lettuce Salad	A random sample revealed a positive result for *Salmonella*	Voluntary recall	Multistate
Taylor Farms Retail	October 19, 2011	*Salmonella*	Various salad blends	A random sample revealed a positive result for *Salmonella*	Voluntary recall	Multistate
Thorntons, Inc.	October 1, 2011	*Salmonella*	Garden and chef salads	Potential cross-contamination of grape tomato linked *Salmonella*	Voluntary recall	Multistate
J&D Produce	December 28, 2010	*Salmonella*	Fresh greens	A positive test linked to *Salmonella* on curly parsley	Voluntary recall	Multistate
Fresh Express	May 24, 2010	*Salmonella*	Various Romaine Ready-to-eat	A random sample revealed a positive	Voluntary recall	Multistate

			salad blends	result for *Salmonella*		
Tanimura & Antle	July 21,2009	*Salmonella*	Romaine Lettuce	A random sample revealed a positive result for *Salmonella*	Voluntary recall	Multistate

On April 14, 2012, Dole Fresh Vegetables voluntarily recalled 756 cases of DOLE® Seven Lettuces salad distributed in fifteen U.S. states *(80)*. A Dole lettuce product tested positive for *Salmonella enterica* during a random collection sample test in New York. The source of contamination was unknown. No illnesses were reported; however, if precautionary actions had not been taken, severe consequences leading to food-borne illnesses outbreak could have occurred *(80)*.

To the best of our knowledge there have been no attributed outbreaks of *Salmonella* spp. associated with lettuce produced hydroponically. However, *Salmonella* outbreaks of alfalfa sprouts grown hydroponically have been reported *(79)*. Other recalls traced back to 4 oz Alfalfa Sprout Cups produced by Arizona Hydroponic Farming LLC of Eloy, Arizona. No illnesses were reported during this episode; however, bacterial contamination of sprouts is well documented. This establishes that contamination occurs in hydroponic environments, and that outbreaks of food-borne illness are possible from hydroponically produced lettuce *(79)*.

Characteristics and Morphology of *Salmonella enterica*

Salmonella is characterized as a motile, non-spore forming, gram-negative, rod-shaped bacterium in the family of *Enterobacteriaceae (37)*. *Salmonella* is categorized into two species, *Salmonella enterica and Salmonella bongori*. *Salmonella enterica* is arranged into 6 subspecies *(37)*. *Salmonella enterica* is further sub-divided into serovars. The Kaufmann-White typing

scheme classifies over 2,579 serovars, based on their reactivity to surface LPS and flagella antigens *(37)*.

The majority of the serovars in *Salmonella enterica* subspecies enterica are classified as non-typhoidal *Salmonella* because they result in symptoms associated with gastrointestinal illness. However, a few serotypes, the most notable being *S. Typhi*, are associated with much more dangerous systemic infections referred to as Typhoid fever *(37)*. With the exception of *S. Typhi*, *Salmonella enterica* is normally found in the intestinal tracts of reptiles, animals, and birds and is transmitted to humans when feces contaminate food or water *(12)*.

Young and/or elderly with weak immune systems, or who are immuno-compromised due to chronic illness, HIV or chemotherapy for cancer, are at a higher risk of acquiring *Salmonella enterica* infections *(37)*. Fewer than 100 cells of *Salmonella enterica* may be sufficient to cause disease in humans, though the infectious dose is dependent on age, immunization status, and epidemiology of various serotypes of *Salmonella*, which vary in their virulence for humans *(14)*.

Salmonella enterica can pose serious public health concerns, particularly in the produce industry, because it has the ability to persist at low temperatures *(18)*. *Salmonella enterica* has been associated with outbreaks of human disease from commercial scale field and greenhouse produce production.

FoodNet analyzed trends of food-borne diseases between 1996-2010 and results show *Salmonella enterica* infections have not declined in 15 years, and steadily increased between 2006-2008, increasing public health concerns *(26)*. Different types of *Salmonella* genotypes and a variety of reservoir contamination are some of the major challenges to reducing the numbers of *Salmonella* infections that occur in a wide variety of foods *(26)*.

Pre-Harvest Handling and Potential Reservoirs of Contamination

Leafy greens can be contaminated at any point during growth, transport, or handling. Reducing pre-harvest contamination is an important line of defense in decreasing numbers of produce-associated food-borne illnesses. Optimizing pre-harvest treatments can improve the microbiological safety of leafy greens. Prior studies have extensively demonstrated the transmission of bacteria to produce in a variety of ways including animal feces, soil contamination, improperly composted manure, sewage, runoff from pastureland, storm related events such as floods, and contaminated irrigation water (*16,29,59,71*). It is important to establish and maintain good human hygiene practices to reduce leafy greens-associated outbreaks.

The use of manure and other fertilizer materials derived from animal waste, water quality for irrigation and washing, and the hygiene of workers and facilities are areas of emphasis to reduce pre-harvest contamination (*12*). Gallegos-Robles et al. (*34*) assessed several cantaloupe farms and tested 28 samples of cantaloupe. Of those samples, 43% (12 of 28) tested positive for *Salmonella enterica,* which can survive in soil, irrigation water sources, and on the hands of workers who handle cantaloupes (*34*).

Ponds, rivers, streams, municipal water, and reclaimed water are common sources of irrigation water used for produce production *(74).* Irrigation water can introduce pathogens from animal fecal matter directly or indirectly on production fields. The contaminated feces can be activated by water and moved through irrigation systems (*5,53*).

A 2006 outbreak of *Escherichia coli* O157:H7 in bagged spinach was traced back to a California production site (*43*). The outbreak strain matched isolates from feral swine, cattle feces, surface water, sediment and soil approximately one mile from the spinach production

fields. Livestock carriers of *Escherichia coli* O157:H7 feces served as vehicles of transmission to water and soil contamination *(43)*. Cooley et al. *(27)* conducted an assessment of *Escherichia coli* O157:H7 for 19 months in a Salinas Valley, California watershed characterized by surface water, creeks, and streams.

Fifteen of 22 water sources in the watersheds sampled tested positive for *Escherichia coli* O157:H7. Watersheds linked with *Escherichia coli* O157:H7 frequently contained cattle. In addition, heavy rain or flooding events caused an increased flow rate that contributed to higher incidences of *Escherichia coli* O157:H7 *(27)*.

Irrigation methods can impact the degree of microbial contamination and its survival on leafy greens. Solomon et al. *(71)* showed that a greater number of lettuce samples tested positive for *E. coli* O157:H7 when applied through spray irrigation compared to drip irrigation. This was a particular concern where water sources were limited and reclaimed recycled water was an attractive alternative method of irrigation water *(71)*. Routine sampling and maintaining potable water sources was highly recommended to reduce food-borne associated outbreaks *(76)*. This study reinforces the importance of choosing premium quality irrigation water and knowing its origin and distribution.

Prior studies have demonstrated the persistence of *Salmonella* Typhimurium on leafy greens in the fields treated with contaminated composted manure and irrigation water *(51, 70)*. Lapidotand et al. *(51)* revealed that *Salmonella* Typhimurium has the ability to persist in favorable environmental conditions for 161 days in the manure composts applied as fertilizer for commercial lettuce production. A maximum amount of time should be allowed between the final application of properly composted manure and harvest, to kill pathogenic bacteria in the field, reducing the risk contamination *(41)*.

Pre-harvest prevention strategies are often implemented to maintain disease control. Suggested strategies such as fencing the production fields to provide barriers to domestic and wild animals, selection and placement of production fields to be away from high traffic of domestic and wild animals, and ensuring the production fields have enough distance from irrigation water sources to prevent contamination. It is important to establish clear and marked barriers to prevent cross-contamination, and reducing human activities may prevent the distribution of food-borne disease.

Post -Harvest Packaging and Handling and Potential Reservoirs of Contamination

Post-harvest causes of contamination may include one or more of the following: poor worker hygiene, improper sanitation of equipment, transport containers and transportation systems, cross-contamination, wild and domestic animals, insects, irrigation water, ice, temperature abuse during storage, packaging, or preparation *(12, 19)*. Transportation systems such as trucks that were previously used to transport animals or meats and then improperly sanitized could cross-contaminate produce during transport from farms to distributors *(73)*. The economic viability of the food industry and consumer safety relies heavily on prevention of contamination *(68)*.

After harvest, further processing of leafy greens such as slicing, shredding, squeezing and peeling, can provide opportunities for contamination to occur via the infected hands of the handlers, contaminated water for final washing or rinsing, cross-contamination from one item to another or equipment. Water is a recognized vehicle that allows pathogens to be distributed from one area to another. During post-harvest processing, clean water baths used to wash and rinse produce are vital to reduce cross-contamination. The contaminated water could promote cross contamination across commodities in the same facility *(73)*. Wachtel et al. *(88)* concluded that

adding one inoculated lettuce leaf to 350 g of chopped dry lettuce resulted in 100% contamination of the lettuce leaves, testing positive for *E. coli* O157:H7 even when stored in water at temperatures of 4˚C and 25˚C for 20 hours. *E. coli* can continue to increase in numbers due to the release of nutrients from produce at storage temperatures above 8˚C *(88)*. The water availability, temperature, tissue damage, nutrients, and native microbiota are all factors that can sustain pathogens associated with produce *(62)*. The risks for amplification of pre-existing pathogens on fresh produce include release of nutrients due to mechanical injury and other post-harvest handling.

Cross-contamination is a recognized source of transferring pathogens to leafy greens that potentially could lead to food-borne outbreaks. Contamination may be present through inadequate hygiene, both of workers hands and of processing facilities and equipment. Emphasizing the importance of hand washing and maintaining clean processing facilities is critical to reduce the risk of food-borne outbreaks. For example, 41 cases of *Salmonella* Bovismorbificans were linked to cross contamination from an inadequately sanitized cutting wheel used to shred multiple heads of lettuce *(72)*. In another shredded lettuce-associated outbreak, the outbreak strain of *Shigella* was isolated from an infected worker and found in the processing plant *(28)*. Moore et al. *(56)* showed that serotypes of *Salmonella* and *Campylobacter jejuni* can both be transferred from contaminated stainless steel surfaces to wet or dry lettuce even when the surface contamination occurred 2 hours previously *(56)*. In Oklahoma, an outbreak of Campylobacteriosis was traced back to cross-contamination of lettuce by contaminated utensils and hands used to process raw chicken *(25)*.

Molecular Mechanisms of Attachment of *Salmonella* spp. to Plants

Understanding the preferred mechanisms of attachment of pathogens, particularly *Salmonella enterica*, will enhance intervention strategies to prevent outbreaks associated with leafy greens. Food-borne pathogens have the capacity to thrive outside their hosts by adjusting to their new surroundings. Variation among cultivars, different genotypes, physiological state and type of fruit or vegetable, can influence the microbial communities associated with produce. Klerks et al. *(46)* proposed that *Salmonella enterica* serovars interact differently with different lettuce cultivars, which greatly influence their survival. Various ecological niches of pathogens can vary depending on surface morphology, metabolic behavior, and tissue arrangement of the leafy greens *(11, 52)*. Brandl et al. *(16)* analyzed the effects of leaf age as a risk factor for distribution of *E.coli O157:H7* and *Salmonella* associated with lettuce during pre- and post-harvest stages. The study showed both pathogen populations increased 16 to 100 fold on young leaves compared to older leaves, respectively, when stored at 28˚C. The analysis of exudates collected from leaf surfaces of different ages showed that young leaves were 2.9x richer in total nitrogen and 1.5x richer in carbon compared to the older leaves *(15)*. In contrast, Kroupitski et al. *(49)* demonstrated that older lettuce leaves were the preferred choice of attachment of *Salmonella* Typhimurium compared to younger leaves. It was suggested that the degree of attachment correlated to the lettuce surface morphology, which also changed with leaf development. In addition, it was observed the preferred attachments on surfaces were closer to the petiole, 7.7 log CFU/g, and on the lower surface of the leaf *(49)*.

Cellulose, fimbriae, and O-antigen capsule are important non-specific bonding attachment mechanisms of food-borne pathogens to leaf tissue surfaces *(6, 7, 18)*. Non-specific attachment to plant surfaces in *Salmonella enterica* is encoded by genes including *yihO, bcsA,*

rpoS, and agfD (6, 7). The gene *rpoS* is recognized as a general stress regulator for *Salmonella enterica.* It promotes environmental survival and is important for biofilm formation, a strategy that may promote survival on leaf surfaces. Other biofilm formation genes are also important for plant colonization including *bcsA,* which triggers the production of cellulose, *yihO,* which activates the glucuronide transporter required for capsule transport to the cell surface, and gene *agfD,* which regulates the production of cellulose and O-antigen capsule contributing to attachment *(6, 7).* Kroupitski et al. *(50)* demonstrated a polystyrene plate model to evaluate biofilm production of various *Salmonella enterica* serovars on intact and cut lettuce leaves, and discovered strong biofilm producers attach better than weak biofilm producers. Patel et al. *(63)* showed that *Salmonella* Tennessee and Thompson generated stronger biofilm formation on lettuce leaf and cabbage leaf surfaces compared to *Salmonella* Newport, Negev or Braenderup, suggesting that attachment can be influenced by serovar characteristics. Overall, the results showed *Salmonella enterica* preferred attachment to lettuce rather than cabbage at intact and cut surfaces *(63).*

Survival and growth of human pathogens can also be influenced by the presence of other microorganisms. Wells et al. *(90)* observed a positive correlation between leafy greens infected with bacterial soft rot pathogens and those that were also positive for presence of human enteric pathogens, particularly *Salmonella enterica*. Bacterial soft rot is a post-harvest plant disease associated with poor handling, storage abuse, or poor sanitation. Results revealed that out of 401 samples affected by bacterial soft rot, 66% tested positive for *Salmonella.* In comparison only, 30% of 402 healthy samples tested positive, indicating a likely association between bacterial soft rot and *Salmonella*. Post-harvest plant disease such as rotten tissue can provide nutrients that would be available to bacterial microorganisms helping them to grow and persist *(90).*

Previous studies have proposed that *Salmonella enterica* serovars can colonize the rhizosphere, depending on cultivar types, produce types, different genotypes and physiological state of the produce influenced the route of preferred colonization mechanisms *(7, 10, 11, 45-47).* Sugar sources, such as fructose, in the root exudates attract *Salmonella* making colonization of the rhizosphere more likely *(10, 47).* A better understanding of attachment mechanisms used by pathogens, particularly *Salmonella enterica*, will improve food safety by helping to implement intervention strategies to reduce bacterial contamination of leafy greens during hydroponic production.

Food Safety and Prevention Strategies

Proactive food safety should include preventative measures with a reduction in food-borne illness outbreaks as the ultimate goal. Leafy greens sanitation practices should start at the beginning of the production cycle with seed germination and continue through subsequent production stages such as growing, harvesting, post-harvest handling, transportation, and processing and preparation.

GMPs (Good Manufacturing Practices), GAPs (Good Agricultural Practices, and HACCP (Hazard Analysis and Critical Control Point) are critical and fundamental preventative methods to ensure the microbiological safety of produce by limiting contamination by human pathogens. HACCP focuses on food safety and implements preventive controls that can eliminate or minimize food safety hazard risks posed by microbiological, chemical, and physical factors *(8).* Producers and management should consider incorporating a HACCP plan for all post-harvest handling processes, including those in the greenhouse and packing house. Currently, the US Food and Drug Administration, FDA, does not require producers to implement the HACCP systems for leafy greens that are required for other food commodities.

A joint effort by the United States Department of Agriculture (USDA) and the US Food and Drug Administration (FDA) developed voluntary guidelines entitled "Guide to minimize microbial food safety hazards for fresh fruits and vegetables". This document highlights good agricultural practices (GAPs) to reduce field contamination and good management practices (GMPs) to minimize the risk of microbial contamination in fresh leafy greens *(83)*. Specific GAPs guidance has been developed for field-grown lettuce and leafy greens, yet implementation of these practices can only reduce but not completely eliminate microbiological contamination associated with fresh leafy vegetables *(81)*. At this time there are no guidelines or practices required by law regarding post-harvest handling of leafy greens grown hydroponically. Cooperation by U.S. government and producers is strongly encouraged to focus on the improvement of the microbiological quality of leafy greens in hydroponic production. These united efforts to establish practices and risk management practices for hydroponic production are vital to reducing the impact of food-borne illness outbreaks. These actions will ensure consumers a safer supply of leafy greens, which improve public health. Continuation of research on post-harvest contamination by human pathogens and their mechanisms of survival in field and/or hydroponic production setting are needed to develop food safety intervention strategies throughout critical stages of leafy greens production.

In addition to prevention, reducing the spread of outbreaks and educational programs on biological contamination are important factors for maintaining food safety. Educating workers of potential risks and prevention strategies can greatly reduce contamination caused by food-borne pathogens. It is critical that workers in the leafy greens industry and consumers understand the basic principles of food safety when handling raw leafy greens. In addition to proper training, monitoring, and preventative action are needed to maintain food safety practices *(3)*.

Methods of Leafy Greens Sanitation

Proper sanitation methods are an important intervention steps at critical points during production, harvest, or preparation. Using potable water to wash leafy greens can remove loose surface contaminants such as dirt and insects, ultimately lowering the bacterial load by 1 to 2 log CFU/g *(49).* The reduction in bacterial load can help both extend shelf life and improve quality of leafy greens, but does not completely remove human pathogens from contaminated leafy greens *(66)*.

The Food Safety and Inspection Service (FSIS), recommends all water used for post-harvest washing of produce consist of potable water plus chlorine 50 to 200 parts per million of free (available) chlorine at a pH of 6.8 to 7.2 with a contact time of 1-2 minutes to limit cross-contamination during washing and between lots *(13)*. To optimize sanitation, it is important to monitor the pH of the wash water, the concentration of free chlorine, the concentration of inorganic and organic matter, and the temperature of the solution *(13).* Poor sanitation may lead to bacterial contamination of washing systems. For example, wash water used in the preparation of pre-washed bagged lettuce can transfer bacteria from one batch to the next, with the potential of infecting a full day's production of lettuce.

Sanitizer agents reduce, but do not completely eliminate, microorganisms established on food surfaces, making their removal during processing and handling procedures difficult. Bacteria can infiltrate cracks in leaves where they avoid contact with chemical sanitizing agents ineffective. Furthermore, the bacteria can form biofilms with strong attachments to the leaf that prevent surface removal *(18, 31, 66)*. Weissinger et al. *(89)* applied inoculated *Salmonella* Baildon to shredded lettuce to determine the efficiency of chlorine sanitation on bacteria during storage at 4˚C for up to 12 days. Shredded lettuce was inoculated with 3.60 log CFU/g of *S.*

Baildon and immersed into a 200 mg/mL free chlorine solution for 40 seconds, which resulted in less than 1 log reduction, suggesting this treatment was ineffective for eliminating the pathogen *(89)*. Zhang et al. *(91)* reported that the most effective surface disinfection methods for inactivating *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* from lettuce leaves and roots was dipping in 80% ethanol for 10 seconds followed by immersion in 0.1% $HgCl_2$ for 10 minutes. However, these treatments are much too harsh and use compounds that are dangerous for human consumption *(91)*. New strategies to reduce spoilage and to inactivate pathogens using ozone *(12)*, gamma irradiation *(12)*, and hydrogen peroxide treatments have been effective but are not currently used in the industry *(91)*.

Hydroponics

Hydroponics technology has increased in popularity for greenhouse production of vegetables, particularly leafy greens. In Virginia, Red Sun Farms will build hydroponic productions on 45 acres in the New River Valley Commerce Park and hope to create 205 jobs over five years *(30)*. Reports revealed hydroponic production generated $544 million in revenue and expected to have an annual growth of 7.8% in US *(40)*. Hydroponics is a production method used for growing plants in plant-nutrient supplemented water without soil *(36)*. This alternative agricultural practice has the potential to reduce land requirements for vegetable production by at least 75% and to reduce single use irrigation water by 90%, appealing to producers with limited land space and access to water resources *(15)*. Recaptured and recycled drainage water yielded a 33% reduction in water production of cucumbers without reducing yield. This creates an attractive solution of using recaptured recycled water to reduce environmental impacts of drainage water discharges, thus reducing pollution issues. Using recycled water can be appealing to producers by saving revenue and becoming more eco-friendly. This eco-friendly

strategy reduces water and sewage costs for aquaculture producers while producing a horticultural crop with its own economic value *(36)*. However, despite some of the attractive features, a major downside include the potential for roots to be contaminated as roots are constantly soaked in recirculating water system. This could increase potential risk of root access by providing nutrients to harmful human pathogens including 17 species of *Phytophthora*, 26 of *Pythium*, 27 genera of fungi, 8 species of bacteria, 10 viruses, and 13 species of plant parasitic nematodes *(39,55, 65,75)*.

Finding a reliable source of fresh water is challenging for conventional producers worldwide. The limited water resources are costly to sustain production, thus increasing the use of low quality water for irrigation on production fields is attractive. However, surface water may raise the risk potential of food-borne infections *(33,74)*. The U.S. Environmental Protection Agency (EPA) conducted a survey of water quality standards in 2004 in the United States. Analysis indicates 44% of the streams, 64% of the lakes, and 30% of estuaries were below water quality standards for designated uses such as drinking, swimming or fishing *(84)*. Previous studies indicate *Salmonella enterica* can swim small distances and in lab studies can serve as a carrier for contamination of hydroponic lettuce and spinach with *Salmonella (42, 50)*. This is a concern and needs to be taken into consideration when evaluating the effectiveness of sanitation methods while screening for bacterial contamination in water.

Selma et al. *(69)* compared soil and soilless production systems and their impact on microbiological and sensory qualities of 'Red Evasion' (*lollo rosso*), 'Red Oak Leaf' (*Jamai*) and 'Green Butterhead' (*Daguan*) lettuce. It was observed that lettuce cultivated in soilless systems was of better quality; containing higher amounts of phytochemicals, particularly vitamin C compared to soil cultivated lettuce. The lettuce grown in a soilless system had lower coliform

numbers and fewer spoilage associated lactic acid bacteria compared to soil grown lettuce *(69)*. One attractive benefit that soilless system cultivation offers is the precise control of plant nutrients and this could potentially increase the yield of leafy greens. Cash crops that have short reproductive cycles and generate high yields are practical candidates for soilless system *(32, 60)*. However, contamination routes of leafy greens in a hydroponic production is not well understand.

Koseki et al. *(48)* examined two possible routes of contamination of hydroponically grown spinach leaves. *Salmonella enterica, Listeria monocytogenes, and Escherichia coli* O157:H7 were inoculated into the hydroponic system and directly applied to the leaves. Results indicated the ratio of contamination was 6.93 higher on the roots compared to the leaves *(48)*. This suggested the primary route of contamination in the hydroponic system was through the roots rather than direct application of pathogen contamination onto the surface of the leaves *(48)*. Once the main routes of contamination on leafy greens are identified, strategies to prevent or reduce hazards must be implemented at its critical control points throughout the food production chain. This ensures consumers access to a safe food supply of leafy greens and protects the well being of the consumers resulting in an overall lower risk of food-borne illness outbreaks.

Hydroponic production of Butterhead Lettuce

Lettuce (*Lactuva sativa),* a dominant component of salad ingredients for its unique texture and flavor, belongs in the Asteraceae family (formerly Compositae) *(57, 58)*. Lettuce matures approximately 40-65 days depending on cultivar type and growth conditions *(57, 58)*. Lettuce is normally composed of 12 to 20 florets, but as low as 7 or as high as 35 is not unusual *(57,58)*.

Lettuce is a cool season crop with temperatures ranging from 60 -65˚F required for optimum growth *(35,57).* High temperatures result in tip burn, poorly developed heads and low density *(35).* Lettuce flowers when exposed to combinations of high temperature and long day length exposure so these conditions must be avoided to successfully grow lettuce. The coastal valleys of California in particular have ideal conditions for high quality lettuce field production including consistent cool and dry weather, good soils, and a long growing season. Although hydroponic production of living lettuce is increasing in popularity, the Salinas Valley central California coast produces 90% of the nation's lettuce supply *(86).*

Insect infestation such as aphids, leafhoppers, and cutworms are serious problems reducing lettuce quality, and they feed on immature heads of lettuce. In addition to pest infestation, viruses and diseases are serious problems that reduce the yield and quality of lettuce production. Some of the well-known viruses and diseases are downy mildew, lettuce mosaic, big vein virus, and corky root bacteria disease *(8, 35, 57).* Most of these are controlled by growing resistant cultivars and purchasing certified seed *(8, 35, 57).* In field production, 'Crisphead' lettuce is extensively grown and as a result consumed because of two distinct characteristics traits: long distance shipping and long shelf life quality. It has lower nutritional quality compared to other leafy lettuce such as 'Butterhead' (*35, 58).* 'Butterhead' is the dominant type used for living lettuce packaging. The yield of lettuce is the product of head weight and the number of leaves harvested per unit area, which is important for producers. Besides cultivar differences, yield can be influenced by several factors such as plant density, head density, cultural practices, disease/insect control, and the percentage of plants harvested by the shipper *(35, 58).*

Lettuce has a shallow root system and receiving a steady supply of water is critical to optimizing growth and development. Drought stress can lead to leaf tip burn that is caused by

calcium deficiency. Too much water irrigation can lead to loose head formation or splitting of lettuce heads, which results in a reduced storage life and quality *(35,58)*. Having a shallow root system allows a quicker turnover rate of production compared to field production since nutrients are constantly supplied and available. However, it is unclear how these unique conditions in hydroponic systems will influence the survival of human pathogens in the system.

Improvements in post-harvest handling will extend shelf-life quality, maintaining color and appearance, texture, flavor, and nutritional value of leafy greens.

Post-Harvest Handling of Butterhead Lettuce in a Hydroponic Production

Lettuce is a highly perishable commodity, therefore maintaining proper temperature and relative humidity ensures best quality during shipping and storage. The shelf life of butterhead lettuce can be extended to 15-18 days by keeping the roots intact and packaging within a plastic clamshell (*8, 35, 58)*. The majority of butterhead lettuce is hand-harvested since it is very prone to bruising and damage, which increases its perishability. Vacuum cooling, an effective method of removing heat from field grown lettuce optimizes shelf-life by maintaining proper temperature and relative humidity *(8, 35, 58)*; however, due to the high costs associated with the vacuum-cooling equipment, post-harvest cooling of greenhouse lettuce is often using forced air-cooling.

GMPs (Good Manufacturing Practices) and GAPs are critical, fundamental, and preventative methods to ensure microbiological safety of living lettuce in a greenhouse setting. Currently there are no accepted guidelines of GAPs (Good Agricultural Practices) designed for hydroponic production of butterhead lettuce. Leafy greens sanitation should start from the early stages of germination and should continue throughout the production stages. These insights will be valuable in developing agricultural practices for post-harvest safety of butterhead lettuce. The following recommended guidelines on harvesting living lettuce are simple procedures but vital to

maintaining the leafy greens at its premium conditions in the hopes of maintaining good shelf life stability: 1) Harvest in the morning and in the cool parts of the day. 2) Pack into cool crates and protect from direct sunlight. 3) Transport in covered vehicles. 4) Cool storage facilities to store leafy greens are important to remove heat *(57).*

Intact lettuce roots systems are sometimes harvested to promote good shelf life stability. Some, large hydroponic lettuce producers package butterhead with the root system wrapped into a knot (bundled up) beneath the head and both sealed in a plastic bag or box (*57).* The bagged or boxed lettuce is packed into crates/trays to be transported to the market. It is important that the bagged lettuce is packed carefully to avoid bruises and leaf damaged during traveling *(57).* Different post-harvest handling practices vary by the size of the hydroponic lettuce production.

Hydroponic butterhead lettuce grown for a local farmers market in Blacksburg, Virginia as living lettuce with roots intact and packaged into plastic containers. These pictures were taken by Jessie Waitt, Blacksburg, Virginia in the Spring of 2012.

1. Different growth stages of Butterhead Lettuce 'Charles' from Paramount Seed Company in a Styrofoam floating raft system. As the lettuce matures, they are transferred to a new raft with wider spacing to promote growth.

2. Roots of a living lettuce in a Styrofoam floating raft system. Notice the even distribution of growth hole spacing in the raft.

3. Post-harvest handling of living lettuce. Rockwool, a man-made mineral fiber that supports and promotes root growth for hydroponic production, is removed at the lettuce/roots interface by hand.

4. Dead and excessive roots are trimmed by hand. The remaining living healthy roots are wrapped into a knot by hand.

5. Living lettuce placed in a clear plastic clamshell ready for sale. Please notice the depression in the bottom of the container, this maintains root health since the moisture condenses and drips into the depression. This provides water to the roots, which prolongs the shelf life of lettuce.

Typical Production Practices in Small Hydroponic Greenhouses in Virginia

Amber Vallatton, a Virginia Cooperative Extension Agent, discusses her experiences with five different hydroponic lettuce producers as they work towards GAPS certification through an e-mail interview *(87)*. One of the questions I specially asked "What are the step by step post-harvest handling procedures you have observed used by hydroponic lettuce producers in Virginia?" Below are the 5 different settings of hydroponic lettuce producers in Virginia.

Example 1: A producer uses a vertical stack hydroponic method and grows roots within a vermiculite mixture growing media. At harvest, living lettuce is placed directly into a Rubbermaid™ wheelbarrow then transported to a separate packing area. Workers are expected to follow good hygiene practices by washing hands before wearing nitrile gloves during handling and packing. Gloves are changed between harvest and packing areas and replaced frequently, particularly when they become soiled. Knives are washed with soap and water, soaked in oxidate, aboard spectrum bactericide/fungicide, following label instructions (3/4 oz). Roots were aseptically removed on stainless steel table tops, and lettuce leaves are placed in clamshells. Once packaged, they were placed into a cold room (coolbot, http://www.storeitcold.com/),

which is used to cool the lettuce to 41˚F. Lettuce is transported in coolers with ice to suppliers and sanitized with oxidate between transportation to prevent cross-contamination.

Example 2: Butterhead lettuce can be grown on floating raft system with Styrofoam in a rockwool media substance. At harvest, handlers pull out the whole lettuce head including roots, and place them into blue Rubbermaid™ bins with water in the bottom. It is unclear if the handlers wear gloves during harvest or packaging. Clamshells are not used for packaging. Living lettuce heads are sold as intact with roots. Living lettuce is transported to suppliers in bins.

Example 3: Butterhead lettuce may be grown in a deepwater, floating system in a greenhouse. However, handling has not been observed but producers market living lettuce with roots removed through road-side farmer's markets.

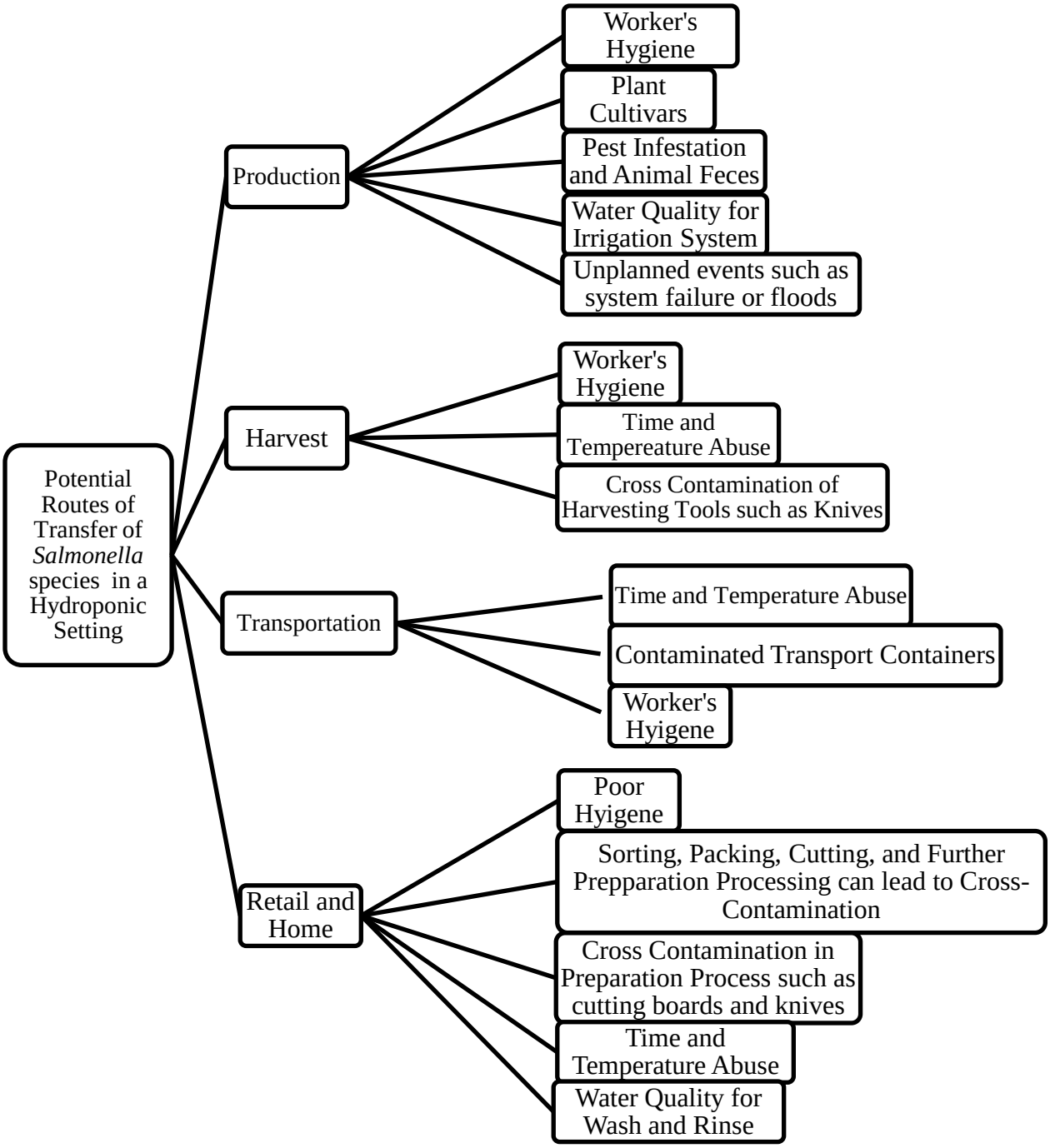
Example 4: Living lettuce can also be produced using a NFT system in an automated greenhouse system where developing heads move on trays from one side of the greenhouse to another according to their growth stage. These hydroponic systems have gutters but not sumps. Gutters are soaked in oxidate for 24 hours to kill algae buildup (algae buildup can reduce the quality of lettuce) between plantings, but the rest of the system is not cleaned. Handlers wore gloves during harvest, but the harvest procedures were not observed. In a separate packaging house, packing tables are cleaned with hydrogen peroxide before placing lettuce into plastic clamshells. Packaged lettuce are placed in walk-in coolers to remove greenhouse heat for at least 24 hours before transport to retailers in coolers on ice. Coolers are sanitized after each batch of

lettuce is transported. This production is currently supplier to farmers' markets and local groceries.

Example 5: Butterhead lettuce can also be produced using simple homemade NFT hydroponic systems in greenhouses constructed using materials from a local hardware store. It is unknown if gutters are sanitized between plantings or what sanitation methods were using during harvest or packaging. Living lettuce including roots were sold directly to consumers.

These variations of post-harvest practices will be valuable in developing safe agricultural practices guidelines for hydroponic production of living lettuce. Practices designed to maintain food safety ensures consumers access to a safe supply of leafy greens, reducing the risk of food-borne illness outbreaks *(87).*

Table 2.4: Potential routes of transfer of *Salmonella enterica* in a hydroponic production.



Glo Germ™ as a Surrogate for Identification of Post-Harvest Cross-Contamination

Precautionary screening for bacterial contamination associated with single-food commodities is an important practice to prevent multistate food-borne outbreaks of illness. If protective measures are not taken, severe consequences, such as legal action against producers or food handlers, can result when contaminated vegetables are sold that cause serious food-borne illnesses outbreaks.

In industry, leafy greens samples are selected at random for testing. *FDA's Bacteriological Analytical Manual (BAM)* is contains standard guidelines for microbiological analyses of food to assess safety and prevention of contamination *(82)*. According to the *FDA's Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella*, the multistep standard guidelines that should be used in industry production to routinely screen for contamination are as follows: 1. Aseptically weigh 25 g of leafy greens into a sterile flask, add 225 ml lactose broth and mix thoroughly, 25 times clockwise and 25 times counterclockwise. 2. Leave the contexts in the flask for an hour at room temperature, measure the pH and adjust to 6.8 ± 0.2 with 1N NaOH or 1N HCl. Incubate at 35º ± 2.0º C for 24 hours. 3. Then transfer appropriate volume of contexts to appropriate selective enrichment media and incubate plates at 35º ± 2.0º C for 24 hours. 4. Read and record presence of colonies such as *Salmonella enterica*.

Screening leafy greens to detect positive *Salmonella enterica* or other bacteria is vital to prevent a food-borne illness outbreak. Poor detection of contamination is a liability risk and appropriate critical actions should be set into place to prevent the chain reaction of contamination throughout a facility or phases of a supply chain *(82)*. Therefore, improving the accuracy of testing for *Salmonella enterica* and other human pathogens on leafy greens is important. One potential method to improve screening of *Salmonella enterica* is by obtaining samples from

specific production regions. This information is critical for successful screening because it allows handlers to see what the potential risk for contamination is throughout a facility.

Direct screening from specific production regions can successfully identify human pathogens on leafy greens and potentially stop an outbreak of food-borne illness from occurring. However, there are challenges in recovering contaminated leafy greens in terms of product age, region of production, limit of detection, specific cultivars, environment, and the resources available. Various examples are given below to demonstrate the difficulty in recovering pathogens from contaminated leafy greens.

Jacobson et al. *(42)* investigated the three preparation methods, soak, stomach, and blend, for the best recovery of *Salmonella enterica* identified from leafy green samples using the *FDA's Bacteriological Analytical Manual (BAM) Salmonella* culture method. Analysis showed 344/540 were *Salmonella enterica* positive when using the soaked method, 293/540 linked to positive *Salmonella* by using stomaching method, and 232/540 by blending method *(42)*. This suggests preparation via soak method is more likely to detect *Salmonella enterica* from leafy greens when compared to other common preparation methods *(42)*. This study suggests that the soak method may optimize the recovery of *Salmonella* from leafy greens.

Previous studies have investigated the potential risk associated with leaf age in the contamination of lettuce with *E. coli* O157:H7 and *Salmonella enterica (16, 49).* It was demonstrated that both pathogens achieved about 10-fold greater numbers on young leaves compared to middle leaves at pre-harvest and post-harvest stages on wet leaves at 28˚C. Leaf age and leaf region should be taken into consideration when screening for biological contamination of lettuce *(16, 49)*.

Another factor that can complicate screening is the variation of microbial load across seasons. Prior studies have demonstrated microbial loads associated with post-harvest leafy greens are influenced by seasons *(2, 20)*. Statistical analysis revealed that Summer and Autumn contain higher microbial concentrations compared to Winter and Spring seasons. These variations in microbial loads could be impacted by drought, warm/cold temperature, moisture content in the air, humidity, and rainy seasons *(2, 20)*. Therefore, it is important to monitor and consider weather conditions when screening samples. Geographic location can impact microbial populations in addition to seasons of the years.

CONCLUSION

Further efforts are necessary to understand the preferred modes of contamination and appropriate intervention needed to reduce the impacts/risks of food-borne outbreaks. An understanding of how bacterial contaminations occur will enhance the microbiological safety of leafy greens in a hydroponic production setting. Post-harvest contamination during handling is a potential source and understanding the risks of handling leafy greens is critical to maintaining safe food practices. Producers should identify the risks associated and implement an action plan to reduce microbial contaminations at its critical points. The identification of risks and development of a plan should be documented and executed to ensure preventive methods that are most effective since washing cannot completely eliminate bacterial contamination. The importance of contamination prevention must be emphasized at all stages of production including harvesting, processing, storage and preparation of leafy greens; this contributes to the safety of leafy greens and economic value of the food industry. It also ensures consumers access to a safe food supply of fresh leafy greens and protects the well being of the consumers resulting in an overall lower risk of food-borne illness outbreaks. Assessing the continuation of reports of

food-borne disease outbreaks is critical towards the success of current and future prevention strategies. These insights will be valuable in developing safe agricultural practices for pre- and post-harvest of leafy greens in a hydroponic production. Hydroponics can function as an alternative agricultural practice that can be sustainable for the production of leafy greens.

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CHAPTER 3: GLO GERM™ AS A SURROGATE FOR IDENTIFICATION OF POST-HARVEST CROSS-CONTAMINATION ON LIVING LETTUCE

ABSTRACT

Fresh produce has been associated with outbreaks of Salmonellosis with increasing frequency. Pre-harvest risk factors have been identified for contamination of field-harvested lettuce; however, the transmission of human pathogens to hydroponic lettuce is not well understood. The purpose of our study was to investigate the use of Glo Germ™, a fluorescent microsphere solution, as a method to visualize cross-contamination from worker hands to the edible tissue of living lettuce. Glo Germ™ solution was combined with *Salmonella enterica* serotype Enteritidis and applied to handlers' hands to identify areas of contact between the solution and the lettuce, providing information about potential for cross-contamination.

The effectiveness of current sampling strategies for detection of *Salmonella* were evaluated by comparing randomly selected leaves under blacklight for Glo Germ™ presence. Living lettuce heads were sequentially processed using typical handling practices, leaves were randomly selected from different sections of the head, homogenized in peptone water, and *Salmonella* were enumerated through serial dilution and plating onto XLT-4 agar. Edible leaves with multiple glowing areas activated under blacklight were selected and processed using the method described previously. On average 4.6±0.06 log CFU/g of *Salmonella* were removed from leaves selected under blacklight compared to 4.1±0.09 log CFU/g from randomly chosen leaves (P=0.05). Although our study suggests that the current FDA methodology for selection of leaves from lettuce heads is adequate, we believe that Glo Germ™ has the potential to be used as a

teaching aid to promote safe handling awareness of leafy greens and minimize cross-contamination risks.

INTRODUCTION

Food-borne illness outbreaks associated with consumption of contaminated fresh produce are increasing in frequency. Produce-associated outbreaks reported by Centers for Disease Control and Prevention (CDC) increased from 2 outbreaks per year in the 1970s to 16 per year in the 1990s *(23)*. Food-borne outbreaks associated with leafy greens reported from 1996 to 2005 have increased by 38.6% *(11)*. Between 1973 and 2006, a total of 502 food-borne outbreaks associated with leafy greens were reported, and of those, 35 were attributed *Salmonella (11)*. Between 1998 and 2008, leafy greens were associated with 186,140 illnesses from bacterial agents, 2,367 hospitalizations, and 26 deaths *(20)*. Human pathogens can be introduced at any point throughout the farm to fork continuum, and the scale of contamination can be amplified through post-harvest handling activities like washing, handling and packing *(4, 7, 17, 21)*. Improved risk-based interventions applied to produce harvesting, transportation, and handling are important to minimize risk of cross-contamination in controlled greenhouse settings.

Consumption of "living lettuce", lettuce heads with intact roots packed in plastic clamshells, is increasing in the United States. Typically, living lettuce is produced hydroponically within controlled greenhouses which reduce potential exposure to wildlife and run-off associated contamination. However, other potential sources of contamination do exist, such as cross-contamination from infected handler to the edible leaves. The purpose of this experiment was to investigate Glo Germ™, a fluorescent microsphere solution, as a method to visualize cross-contamination from worker hands to the edible leaves of living lettuce. Glo Germ™ combined with *Salmonella enterica* serotype Enteritidis was applied to the handlers' hands to identify sections of the lettuce head that are more susceptible to handler-associated

contamination. The effectiveness of lettuce sampling strategies were examined by looking at recoverability of *Salmonella* Enteritidis from randomly selected leaves versus leaves chosen using a blacklight as a tool to identify potential cross-contamination. This evaluates the effectiveness of current sampling methodology in industries.

In industry, *FDA's (U S Food and Drug Administration) Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella,* is the current methodology of detecting *Salmonella spp.* on leafy greens *(25)*. Lettuce leaves are randomly selected from the head during routine precautionary screening for human pathogens *(25)*. Current methodology of random selection may be inadequate due to uneven distribution on the head or ineffective screening. Leafy greens are known to carry *Salmonella*, therefore the *FDA's Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella* method's effectiveness for leafy greens need to be reexamined. *Salmonella* isolation from various food commodities has been done using different culture media, incubation temperatures, and sample preparation *(3, 8-10, 12)*. We hypothesize that sampling efficiency might be improved by targeting specific portions of lettuce (outer vs. inner towards the head or bottom vs. top of leaf), which may be more likely to come into contact with hands of handlers. This informative screening has the potential to aid handlers in visualizing potential contamination risks and promote awareness with facilities.

MATERIALS AND METHODS

Bacterial strain and culture conditions.

Salmonella enterica Enteritidis ptvs177 was obtained from Trevor Suslow of University of California, Davis and passed through increasing concentrations of rifampicin to generate resistance to 100 µg/ml rifampicin (Fisher Scientific, Fair Lawn, NJ). Bacteria were stored at -80˚C in tryptic soy broth (TSB; Difco) supplemented with 100 µg/ml rifampicin (TSB-Rif) and

30% glycerol (Fisher Scientific). Bacterial cultures were prepared by sub-culturing from a frozen stock in TSB-Rif 100 µg/ml and incubated at 37˚C for 24 h. Colony morphology and selective indicators were confirmed by plating onto xylose-lysine-tergitol-4-agar (XLT-4; Difco) containing 100 µg/ml rifampicin (XLT-4- Rif) and incubated statically at 37˚C for 24 h. *Salmonella* colonies were identified by their red coloring with black centers. Enumeration of *Salmonella* CFU/g was performed using a 10-fold serial dilution in 0.1% sterile peptone water solution (BPW; Sigma-Aldrich) and were spread plated onto duplicate XLT-4 plates. Plates were incubated at 37˚C for 48 h.

Inoculum preparation and Glo Germ™ solution preparation.

A sterile disposable plastic loop was used to transfer cells from frozen stocks of *Salmonella enterica* Enteritidis to 100 ml TSB-Rif broth. *Salmonella* cultures were incubated at 37˚C for 24 h. *Salmonella* cells in 0.50 ml of TSB-Rif were washed with 0.1% sterile BPW to remove residual nutrients. After final centrifugation at 3,000 x g for 15 min, the cell pellet was re-suspended in 0.5 ml BPW, and Glo Germ™ solution was added for a total final volume of 12.5 ml. A ratio of 1g Glo Germ™ to 11 ml of 0.1% BPW, the culture was re-suspended in Glo Germ™ solution to create a final 6.5-7 log CFU/g *Salmonella*.

Lettuce preparation and sampling methods.

Butterhead living lettuce heads were purchased from a local retail store in Blacksburg, VA and kept at 4˚C for a maximum of 48 h prior to processing. Spic and Span™ latex rubber gloves (long cuff latex) purchased from a local retail store were used to mimic the physical texture of gloves commonly used to handle leafy greens in the production facilities. To determine the potential transference of *Salmonella* from one living lettuce head to another in sequential handling, 1.0 ml of Glo Germ™-*Salmonella* solution was applied via droplets onto

each hand. Once the Glo Germ™-*Salmonella* solution was applied onto the hands, then typical living lettuce handling tasks were performed: lift a head of lettuce by the base, squeeze excess water from roots, wrap the roots in a knot and transfer lettuce head to a plastic clamshell.

Potential transfer of *Salmonella* from a single contamination event was assessed by performing sequential handling on three heads of lettuce with hands inoculated at the beginning of the sequence. Food coloring was used to provide a visual indicator of where gloves tend to make contact with surfaces during post-harvest handling. Results from this experiment informed the application of Glo Germ™-*Salmonella* solution to the gloves during further experimental procedures.

Living lettuce heads (n=3) were sequentially handled, and 25 g of randomly selected leaves were weighted in a stomacher bag and the contents were homogenized for 2 min in 225 ml containing 0.1% BPW and 0.1% Tween80 (Fisher Scientific) solution in a lab blender (Bag Mixer, Interscience, Weymouth, MA). Enumeration of *Salmonella* CFU/g were determined by performing a 10-fold serial dilution in 0.1% BPW and spread plating onto duplicate XLT-4 plates. Plates were incubated at 37˚C for 48 h. The post-harvest handling study was conducted in triplicate, using 3 heads of living lettuce for each trial. Additionally, living lettuce heads (n=3) were sequentially handled and edible leaves with multiple glowing specs activated under blacklight were aseptically removed. Leaves were selected and processed using previously described method. To validate the presence of bacteria that were below limit of detection in plate counts, 1.0 ml of homogenized contents (leaves) from stomacher bag were added to 9 ml of TSB supplemented with rifampicin and incubated at 37˚C for 24 h. After 24 h the culture was streaked for isolation on XLT-4-Rif and incubated at 37˚C for 24 h. Presence or absence of colonies on each selective plate were recorded as positive or negative, respectively. Non-inoculated controls

(n=3) were processed as above to account for contamination or growth of non-*Salmonella* bacteria on the selective media.

Quantification of Glo Germ™ transmission to edible leaves.

Presence/absence of the non-inoculated Glo Germ™ solution on the older and outer leaves were visualized using the Gel Doc UV light box and the average intensity (Average Quantity) within a 30x30 mm area calculated using the Quantity One 1-D Analysis Software version 4.6.5 (Bio-Rad Laboratories, Inc. ™). The average intensity value of the leaf without Glo Germ™ was subtracted from the average intensity value of the leaf with Glo Germ™ to determine the fluorescence only by Glo Germ™. The Glo Germ™ was put into solution and then the smallest detectable amount was evaluated. The duration of fluorescence was also determined over a 24 h period. To improve the distribution of Glo Germ™ within the solution, 1.0% of weight per volume lecithin (0.12 g of lecithin) was added to 1:12.5 ml Glo Germ™ solution and heated to 30˚C before application.

Growth of *Salmonella* ptvs177 in presence of Glo Germ™ solution.

The Bioscreen C™ (Growth Curve, Inc.) instrument was used to determine the growth rate of *Salmonella* in a TSB/Glo Germ™ solution. Wells containing 178.6 µl TSB-Rif, 20 µl, Glo Germ™ solution were inoculated with 1.4 µl of *Salmonella*. Shaking occurred 15 sec before each absorbance measurements ($OD_{580\ nm}$) reading recorded every 15 min for 48 h at 37˚C. Concentrations of *Salmonella* containing 20 µl, BPW, 1.4 µl *Salmonella* and TSB supplemented with rifampicin 178.6 µl, total volume of 200 µl, were pipette into 10 wells to serve as a positive control of growth. Negative controls consisted of growth media to which bacteria were not added. Microsoft Office Excel 2007 software was used to plot absorbance vs. time (hours) to identify and calculate the lag phase, starting phase, exponential phase, slowing down phase,

stationary phase, and the growth rate constant. Growth curves were run in duplicate using 40 wells.

Statistical analysis.

Bacterial counts (CFU/g) were log transformed to approximate normal distribution. The post-harvest sequential handling and selective method study was conducted three times using six living lettuce heads per trial. Statistical analyses were performed using the using statistical software JMP® Pro 10.0 (SAS; Institute Inc., Cary, NC). Fisher's exact two-tailed F test was performed to test for differences in the bacterial counts and growth rate. The sequential handling was statistically investigated by analysis of variance and Student's *t* test. P-values ($P< 0.05$) with α=0.5 were considered significant.

RESULTS

Testing efficacy of sampling plans for detection of *Salmonella* on living lettuce.

The average log CFU/g of *Salmonella* recovered from lettuce leaves chosen using two different sampling strategies were compared. On average 4.6±0.1 log CFU/g of *Salmonella* detected under blacklight and 4.1±0.1 log CFU/g recovered from randomly selected leaves. We observed a small but statistically significant increase in log CFU/g of *Salmonella* recovered when blacklight was used to select leaves (P=0.05) (Figure 3.4). After each sequential handling event, the log CFU/g of *Salmonella* was reduced. Statistically significant was observed between lettuce head 1 and lettuce head 3 (P=0.0486) selected under blacklight and randomized leaves (P=0.0002). The average recovery of *Salmonella f*rom leaves selected under blacklight on lettuce head 1 was 4.8±0.1 log CFU/g, lettuce head 2 was 4.6±0.0 log CFU/g, and lettuce head 3 was 4.2±0.1 log CFU/g. The average recovery of *Salmonella* from leaves randomly selected on lettuce head 1 was 4.7±0.1 log CFU/g, lettuce head 2 was 4.1±0.1 log CFU/g, and lettuce head 3

was 3.4±0.1 log CFU/g (Figure 3.5). Growth rates (μ=0.79) of *Salmonella* in the presence of Glo Germ™ solution at 37˚C were not significantly different from the control. However, the overall final yield (maximum OD) of Glo Germ™ without the presence of *Salmonella* (OD 580_{nm}= 1.1) was greater than Glo Germ™ containing *Salmonella* (OD 580_{nm}= 0.8). This suggests Glo Germ™ solution may influence the overall amount of *Salmonella* at 37˚C. Non-inoculated controls (n=3) yield no counts.

Use of Glo Germ™-*Salmonella* solution to visualize transfer from gloves to living lettuce.

Prior to applying Glo Germ™-*Salmonella* solution, food coloring was used as a visual guide to determine where the gloves came into contact during post-harvest handling of living lettuce. These results led to a better application of Glo Germ™-*Salmonella* solution on the gloves to assure contact was made with the solution. Food coloring was visible on the roots, base of the lettuce head and tips of leaves (Figure 3.1). Blacklight revealed distribution of small droplets of Glo Germ™- *Salmonella* solution on the roots, and over the outer leaves and across the top of the lettuce heads, including areas that were not directly touched by the handler (Figure 3.2). The use of the average intensity function of the Quantity One program was insufficient to quantify the amount of transference from workers hands to leaves. The readings were inconsistent and frequently the average intensity of the background, non-inoculated leaves prior to application of Glo Germ™ solution was greater than that measured for the leaves that were inoculated with the Glo-germ solution, in addition the inoculated leaves appeared brighter to the researchers (Figure 3.3).

DISCUSSION

Glo Germ™ is a useful tool for monitoring *Salmonella* transmission routes and assessing hygiene practices and identifying control points during post-harvest handling. In this study,

simultaneous application of Glo Germ™ and *Salmonella* was used to visualize the sections of lettuce that are subject to contamination via handler's hands. We observed only a small, 0.5 log CFU/g of Salmonella on leaves that were selected with aid of blacklight to visualize potential contamination compared to leaves that were chosen at random. This supports our hypothesis that the current FDA protocol of randomized leaf sampling in multiple locations in production is adequate. The initial concentration of *Salmonella* applied to the worker hands was 6.5 log CFU/ml, an amount that is likely to be higher than real life contamination events. However, this technique demonstrated that while large numbers 4.0-4.8 log CFU/g were transferred to the lettuce, enough remained on worker hands for transmission to multiple heads of lettuce. While this experiment examined sequential transfer to only 3 heads, the large numbers on head three suggest transfer occurs to even more heads. Taormina et al. *(24)* have demonstrated that *E. coli* O157:H7 can be transferred from knives to lettuce during field coring and a single contamination event can spread the pathogen to 10 heads. Given that the infectious dose of *Salmonella* may be as low as 100 cells of *Salmonella* dependent on age, immunization status, and serotypes, sequential transfer after only one contamination event may pose significant risk to consumers *(1)*. Transfer of human pathogens from human hands to surfaces and to produce has been demonstrated for *Salmonella, E.coli* and *Campylobacter (5, 16, 27).* Wachtel et al. *(27)* examined the cross-contamination risk of 'Iceberg' lettuce associated with contaminated hands and cutting boards. Results revealed 46% of lettuce leaves, including the 25th exposed leaf were contaminated when the leaves were pressed onto a cutting board inoculated with 1.25 X 10^2 CFU of *Escherichia coli* O157:H7 *(27)*. Chen et al. *(5)* reported cross-contamination risk was significant when raw chicken was inoculated with 7 log CFU/g of *Enterobacter aerogenes* and demonstrated that 2.1±0.9 CFU/g was transferred from the chicken contaminated hands to lettuce

leaves and 4.3±0.6 to lettuce from the chicken contaminated cutting board *(5)*. In this study, latex gloves similar in thickness and texture similar to those used in harvest and post-harvest processing of living lettuce. It is possible that the gloves' physical texture and design may improve or inhibit the transfer of bacteria. Additionally, leaks may develop in the gloves, exposing the lettuce to potential hand contamination, emphasizing the importance of good hand washing practices. Latex gloves were less frequently associated with leaks compared to vinyl gloves, another common choice for food service workers *(19)*. Intervention tasks of maintaining good hygiene by hand washing prior to wearing gloves and changing gloves frequently can minimize risk of contamination from hands to ready-to eat produce.

This study compared the effectiveness of two leaf selection strategies on the log CFU/g of *Salmonella* recovered from lettuce inoculated using worker hands. In this study, only a small difference of 0.5 log CFU/g could be detected, suggesting current leaf sampling strategies are adequate to detect worker associated contamination. It was beyond the scope of this study to examine how the recovery method influenced the detection. Previous studies by Jacobson et al. *(12)* compare recovery of *Salmonella* from three preparation methods: soaking, stomaching, and blending followed by enrichment according to methods described by *FDA's Bacteriological Analytical Manual (BAM)*. Recovery of *Salmonella* on the samples was influenced by the initial method used to dissociate the cells from the leaf surface with 344/540 *Salmonella* positive using soaked method, 293/540 *Salmonella* positive using stomaching method, and 232/540 *Salmonella* positive using the blending method *(12)*. In contrast, Burnett et al. *(3)* showed that there was significant difference in recovery of *Salmonella* from 26 various types of produce including fruits, vegetables, and herbs when washing in peptone water, stomaching, or homogenizing were compared. These differences may reflect particular challenges associated with the

morphological characteristics, chemical composition, and pH of leafy greens *(3)*. Further investigation should examine additional preparation methods for recoverability of *Salmonella* isolating from various cultivars and physical characteristic interactions with leafy green leaves.

Physical characteristics such as leaf region, texture and age should be taken into consideration in screening procedures for detection of contamination of lettuce *(2, 14)*. We chose mature and intact living lettuce heads for the focus of our study and our samples were purchased from a local retailer in Blacksburg, VA; therefore we do not know the exact age of the lettuce at the time of inoculation. The typical shelf life of living lettuce is 2 weeks to 3 weeks depending on cultivars *(18)*. There are factors that could influence survival and recoverability of *Salmonella* from lettuce including age, cultivar, how it was produced and processed, and handling by distributor and retailers, all of these are unknown in the current study. Brandl et al. *(2)* investigated the relationship between 'Romaine' leaf age and *Escherichia coli* O157:H7 and *Salmonella enterica* contamination. Both pathogens reached 10-fold higher population sizes on young leaves compared to middle leaves at pre-harvest and post-harvest stages in the presence of water on leaves and temperature of 28˚C *(2)*. This suggests young leaves are more likely to harbor pathogens compared to middle leaves. In addition, nitrogen analysis taken at various leaf age revealed that young leaves were overall richer in total nitrogen and carbon content supporting growth of the pathogens on the leaf surface *(2)*. Conversely, Kroupitski et al. *(14)* proposes that older 'Romaine' leaves were the preferred choice of *Salmonella enterica* serovar Typhimurium attachment compared to younger leaves. It was reported that preferred attachments on surfaces were localized closer to the petiole and on the abaxial side, lower surface of the leaf. In addition, Scanning electron microscopy demonstrated that surface complexity changes as leaf ages, indicating the preferred attachment may coordinate with surface structural texture as it ages

(14). Kroupitski et al. *(15)* demonstrated attachment by various *Salmonella enterica* serovars on intact and cut lettuce leaves, is improved for biofilm producing strains. This is of particular concern as biofilms can be present in the pipes and on gutters used for production of hydroponic lettuce *(15).*

This study demonstrated a wide dispersal of Glo Germ™ across the whole lettuce head and leaf's surface areas; including areas that were not directly contacted by hand were contaminated possibly through contaminated water droplets (Figure 3.2). It is likely that contaminated water droplet transfer further spread the contamination. Therefore, simply removing the outer most leaves may not be the only intervention step to minimized bacterial contamination. To the best of our knowledge, no data are available concerning the interaction of Glo Germ™ and *Salmonella* and it was beyond the scope of this study to examine interaction of Glo Germ™- *Salmonella* solution with lettuce tissue. However, the large size of the fluorescent microsphere particles suggests its behavior would differ compared to a pathogen and is therefore not a good surrogate for survival and detection. Furthermore, since a reproducible method to quantify fluorescence of the Glo Germ™ could not be identified this method does not seem promising for quantification of transfer. Despite use of lecithin as an emulsifier to promote equal dispersion there were inconsistencies in the amount of fluorescence detected before and after application of the Glo Germ™ to the leaf (Figure 3.3). Large amounts of variability were associated with older and outer leaves of same age and region within the leaf. In addition, increased fluorescence over time was observed that may be related to detection of lysed chloroplasts being released, resulting in conflicting average quantity readings influencing the standard curves. The coefficient of determination was r^2=0.68603. Despite, the inability to quantify transfer in this study Glo Germ™ has the potential to visualize risks of contamination

associated with different post-harvest handling practices *(6)*. Vorst et al. *(26)* have used Glo Germ™ powder to identify contact surfaces of deli slicers most likely to be contaminated during slicing, thereby identifying target areas for sanitation *(26)*. Therefore, Glo Germ™ should be considered as a training tool to inform producers and processors of produce of potential areas within the environment subject to cross-contamination, that can be targeted for improved sanitation. Food handler training programs should also consider incorporating Glo Germ™ as part of training programs to help handlers see a visual demonstration that their actions can lead to spread of droplets and bacteria that are not visible to the naked eye. The outcome of this training tool will help workers to understand the important of maintaining good hygiene practices and sanitation practices.

Preliminary studies were carried out to determine XLT-4 containing rifampicin, an antibiotic, as a selective agent allowing discrimination of the inoculated strain from native plant bacteria of the roots and leaves. The ideal media should have no counts when non-inoculated control roots are plated and high recovery of *Salmonella* distinctive colonies from inoculated roots. Results showed successful prevention and XLT-4-Rif selective agar plates were used for isolation of non-typhi *Salmonella* colonies for the duration of this study.

CONCLUSION

In conclusion, Glo Germ™ has the potential to be utilized as a teaching aid to promote safe handling awareness of leafy greens and minimized cross-contamination risks. Our study proposes that the current FDA methodology of detecting contamination is adequate. However, risk management and continuing research effort is a must to provide analysis with methods that are effective for detection and isolating of *Salmonella* from living lettuce. Appropriate post-harvest sanitation practices to prevent contamination remains one of the most important

measures for ensuring the microbiological safety of living lettuce and the well-being of the consumers.

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FIGURES

Figure 3.1: Red food coloring was used as an indicator to visualize where the gloves came into contact with the different parts of lettuce plants during routine packaging of living lettuce.

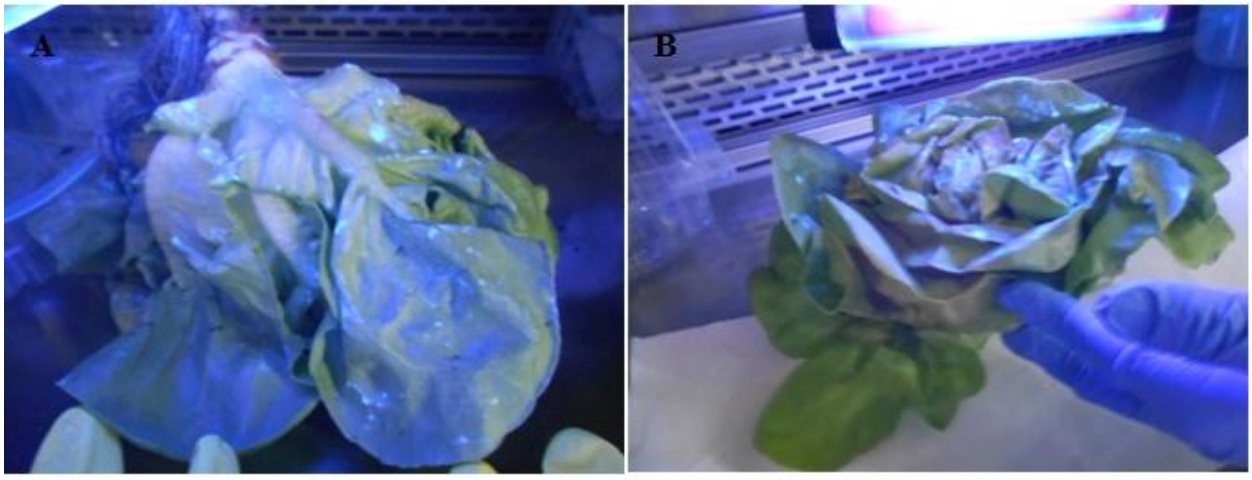

Figure 3.2: Droplets of Glo Germ™ solution on a lettuce head visualized using blacklight.
A) Living lettuce viewed on its side, showing the distribution from bottom to top of the leaf.
B) View of top of the head of living lettuce, showing the wide dispersion throughout the whole surface area and inner leaves.

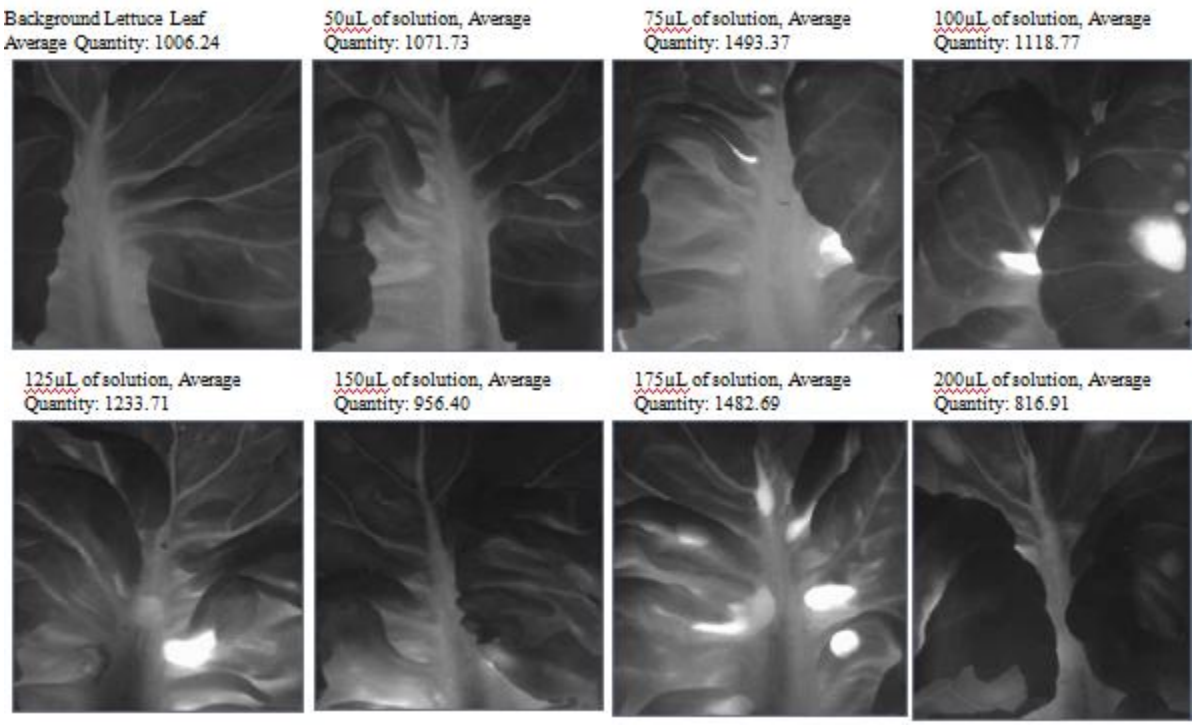

Figure 3.3: Images of older and outer lettuce leaves used to calculate the average intensity (Average Quantity) within a 30x30 mm area. The average quantity value of the leaf without (background) Glo Germ™ was subtracted from the average intensity value of the leaf with Glo Germ™ to determine the fluorescence only by Glo Germ™.

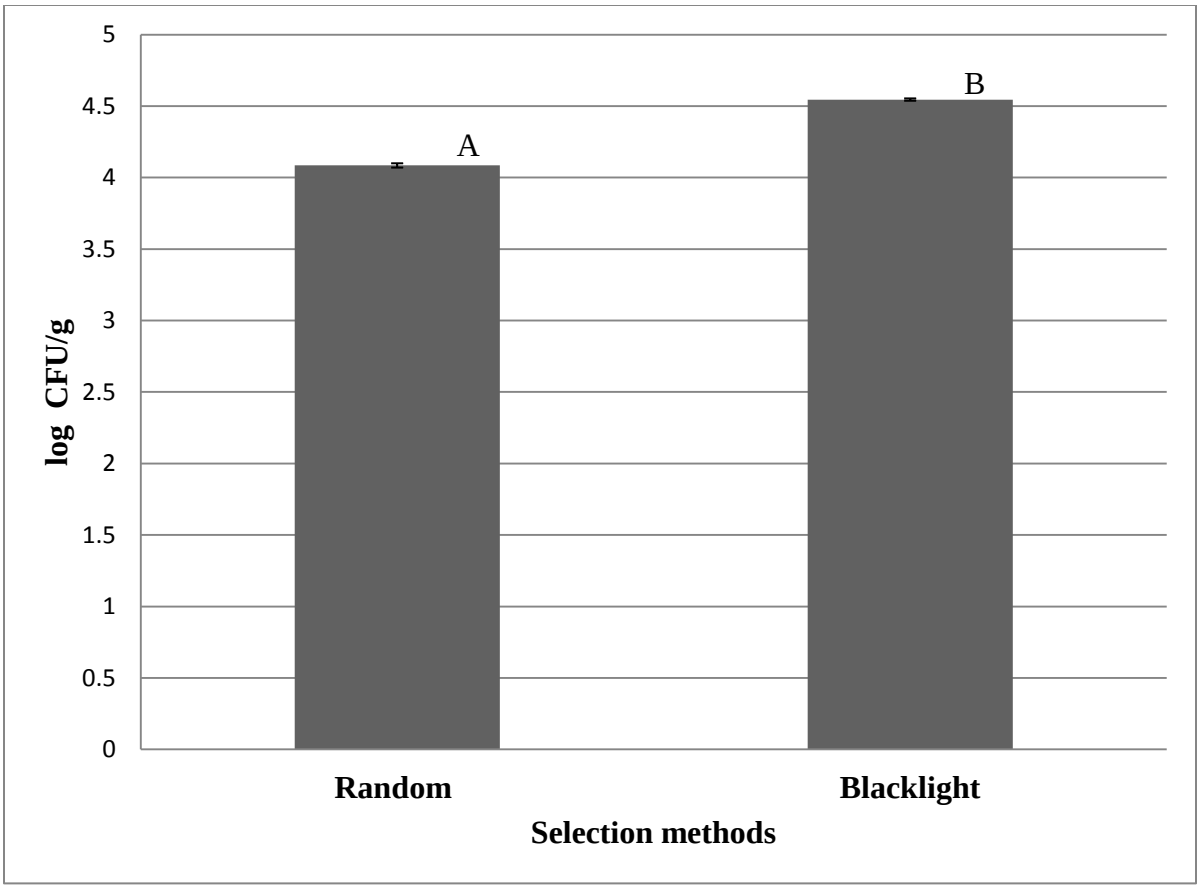

Figure 3.4: Enumeration of *Salmonella* Enteritidis ptvs 177 recovered from living lettuce leaves chosen using two different selection methods: random selection and blacklight to visualize Glo-Germ co-inoculated with the *Salmonella*.

Data shows the average log CFU/g of *Salmonella* and error bars from 3 replicates (n=9) and cells were recovered by plating on XLT4-Rif and incubated at 37˚C for 48 h. Each error bar is constructed using 1 standard deviation from the mean. Samples not connected by same letter are significantly different ($P<0.05$)

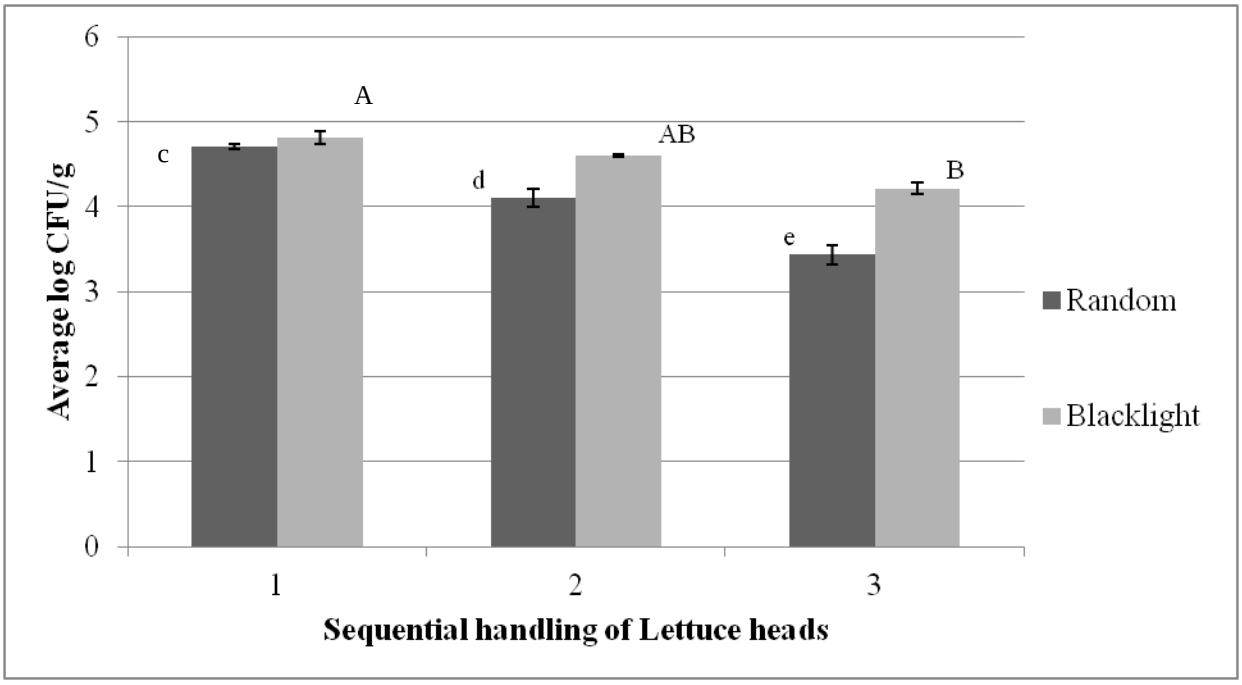

Figure 3.5: The recoverability of *Salmonella* Enteritidis ptvs 177 following a single contamination event from worker gloves followed by sequential post-harvest packing of living lettuce using two different selection methods.

Data shows the average log CFU/g of *Salmonella* and error bars from 3 replicates (n=9) and cells were recovered by plating on XLT4-Rif and incubated at 37˚C for 48 h. Each error bar is constructed using 1 standard deviation from the mean. Samples not connected by same letter are significantly different ($P<0.05$)

CHAPTER 4: POST-HARVEST TRANSFER AND SURVIVAL OF *Salmonella enterica* SEROTYPE *ENTERITIDIS* ON LIVING LETTUCE

ABSTRACT

A number of risk factors for post-harvest contamination of field-harvested lettuce have been described; however, the potential for post-harvest transfer of *Salmonella* in small-scale hydroponic systems is not well understood. The purpose of our study was to quantify the transfer and survival of *Salmonella enterica* Enteritidis from contaminated roots to the leaves of lettuce packaged as "living lettuce" and stored at 4˚C and 12˚C. Living lettuce is packaged within clamshell with intact roots, greatly extending shelf life to 18 days. Butterhead lettuce cultivar '*Buttercrunch*' was grown hydroponically, harvested, and roots were inoculated with *Salmonella* by soaking. The roots were wrapped in a knot and the lettuce placed in clamshells stored at recommended temperature (4˚C) or under temperature abuse conditions (12˚C). Periodically three packages were removed and lettuce destructively processed. Roots and leaves were removed from the head, homogenized in peptone water separately and *Salmonella* enumerated by serial dilution and plating onto XLT-4 agar. On average 5.1±0.00 log CFU/g of *Salmonella* were transferred to the roots from inoculated solution, while only 2.9±0.1 log CFU/g S*almonella* were transferred to leaves from inoculated roots. *Salmonella* persisted but did not grow on leaves when stored at 4˚C or 12˚C for 18-days. Storage at 12˚C was associated with 2 log CFU/g increases in *Salmonella* on roots after 18-days storage (P=0.0002), while 4˚C storage was associated with a decrease of 0.4 log CFU/g *Salmonella* on roots (P=0.0001). This reinforces the need for maintaining temperature control and identifying risks associated with post-harvest handling and distribution.

INTRODUCTION

In the United States, outbreaks of food-borne illnesses are increasingly attributed to fresh produce, with an estimated 46% of food-borne illnesses attributable to produce *(25)*. Produce-associated contamination accounted for 190 outbreaks including 16,058 illnesses, 598 hospitalizations, and 8 deaths between 1973 and 1997 *(30)*. The public health burden associated with contaminated leafy greens has continued to grow with a 38% increase in leafy greens outbreaks from 1996-2005 *(10)*. Between 1998-2008, outbreaks attributable to leafy greens were associated with 186,140 illnesses, 2,367 hospitalizations, and 26 deaths *(25)*. These produce associated illnesses and outbreaks create an annual economic burden of $39 billion *(27)*. *Salmonella enterica* was the etiologic agent in 35/502 leafy greens outbreaks between 1973-2006 in the United States *(10)*. Internationally, outbreaks of *Salmonella* Thompson *(23)* and *Salmonella* Braenderup *(7)* were traced back to contaminated lettuce. Contamination of lettuce and leafy greens can occur anywhere along the farm to fork continuum. Risk prevention and management are critical factors to maintain produce safety and economical value of the food industry *(1)*.

There has been an increase interest in development and implementation of Good Agricultural Practices (GAPs) to reduce produce contamination. GAPs guidance documents are available for field-grown leafy greens but does not address hydroponically produced living lettuce. In the United States, living lettuce with intact roots packaged in plastic clamshells is increasing in popularity. Living lettuce is typically produced in environmentally controlled greenhouses using hydroponic systems. Hydroponic systems incorporate the sustainable agricultural practice of growing produce in mineral supplemented water without soil *(9)*. One attractive benefit of hydroponics systems is the short reproduction cycle within crops that

generate high yields; this makes them ideal candidates for soilless systems, which offer plant nutrients precision that could increase the yields of leafy greens *(6, 22)*. Pre-harvest contamination by insects, wildlife and run-off is reduced in greenhouse hydroponic systems but other sources of contamination remain, chiefly water used for irrigation, foliar application. Post-harvest contamination during harvesting, packaging and washing are associated with increased spread of contamination *(30, 38)*. Water is a recognized vehicle that allows pathogens to be distributed to produce. Studies have demonstrated potential for transfer from water used for overhead irrigation to edible tissue *(11, 13, 18, 29, 33)*. Koseki et al. *(17)* recently identified much greater amounts of transfer of human pathogens to the roots of hydroponically grown spinach plants compared to the leaves *(17)*. This suggests the primary route of contamination in the hydroponic system is through the roots rather than direct application of pathogen contamination onto the surface of the leaves *(17)*. Therefore, handling practices for living lettuce are likely to transfer pathogens from contaminated roots to the edible tissue during packaging. Yet, the survival on intact roots and potential for transfer to edible leaves of living lettuce is poorly understood.

The objective of our study was to quantify the transference of *Salmonella enterica* serotype Enteritidis from contaminated roots to the leaves of a mature Butterhead lettuce packaged as "living lettuce" in a clamshell with intact roots. In addition, the survival of *Salmonella* on lettuce roots and leaves stored at typical retail storage conditions of 4˚C and temperature abuse conditions of 12˚C was determined throughout shelf life. These research findings will provide knowledge about cross contamination in post-harvest hydroponic systems and promote implementation of safe handling strategies and risk management.

MATERIALS AND METHODS

Bacterial strain and culture conditions.

The *Salmonella enterica* Enteritidis strain ptvs177 obtained from Trevor Suslow was passed through increasing concentrations of rifampicin (Fisher Scientific, Fair Lawn, NJ) until resistance to concentrations of 100 µg/ml was achieved. The culture was maintained in Tryptic Soy broth (TSB; Difco, Sparks, MD) supplemented with 100 µg/ml rifampicin (TSB-Rif; Fisher Scientific) and 30% Glycerol (Fisher Scientific) and stored at -80˚C. Bacterial cultures were prepared by sub-culturing from a frozen stock in TSB-Rif and incubated statically at 37˚C for 24 h. Colony morphology and selective indicators were confirmed by plating onto Xylose-Lysine-Tergitol-4-Agar (XLT-4; Difco) containing 100 µg/ml rifampicin (XLT-4- Rif) and incubated statically at 37˚C for 24 h. Positive colonies for *Salmonella* spp. were red with black centers.

Greenhouse growing conditions, harvest, and storage conditions of living lettuce.

Butterhead lettuce cultivar 'Buttercrunch' was grown in hydroponic system to maturity at the Virginia Tech Aquaculture Research Center, Saltville, VA. Seeds were germinated in perlite and transplanted into continuous-flow Nutrient Film Technology (NFT) channels at the 2-leaf stage. Lettuce heads were harvested at maturity after 48 days after transplantation to NFT for trial 1 (n=39) and 58 days for trial 2 (n=39). Differences in times to harvest were associated with the decreased day length associated with the second trial. Lettuce with intact root systems were harvested following typical living lettuce handling practices: 1) lift a head of lettuce by the base using gloved hands, 2) separate roots from neighbor leaving at least 2 inches of roots on either side, 3) squeeze excess water from the lettuce roots, 4) lettuce head with intact roots was weighed, 5) wrap the lettuce roots into a knot form, and 6) transfer the lettuce to a plastic lettuce clamshell (ProducePackaging.com). The living lettuce clamshells were transported on ice to the

Food Science and Technology building at Virginia Tech, and then stored overnight at 4˚C to remove greenhouse heat.

Inoculation of lettuce roots, post-harvest handling, and storage conditions of living lettuce.

The follow morning after harvest (12-16 h), lettuce roots were inoculated with *Salmonella enterica* Enteritidis by immersion for 10 min in nutrient solution within a sterile 500 ml Erlenmeyer glass flask. (Figure 4.1). The nutrient solution contained 1.0 ml of *Salmonella* culture grown statically 24 h in TSB-Rif (OD 580_{nm} = 0.8) in 400 ml 0.1% sterile buffer peptone water (BPW; Sigma- Aldrich). To prevent contamination of leaves during root submersion the lettuce head was encased in a Ziploc® double zipper bag (26.8cm x 27.3cm) with a small hole allowing only the roots to protrude. Handling practices were performed as follows: 1) lift a head of lettuce by the base using gloved hands, 2) the roots were squeezed to remove excess water, 3) wrap lettuce roots forming a knot, and 4) immediately transfer the lettuce with wrapped roots to clamshell container (Figure 4.1).

To examine post-harvest survival of *Salmonella*, two different storage temperature conditions were selected for this study. In trial 1, lettuce packaged in plastic, clear clamshells was stored at 12˚C to simulate temperature abuse conditions that may occur in a holding facility, or in a consumer's kitchen. In trial 2, lettuce was held at 4˚C, the FDA recommended temperature for storage of minimally processed produce *(35).* The study was terminated when signs of reduced produce quality (loss of sample color, loss of cell turgor, tissue weeping and the development of sliminess) were observed.

Microbiological sampling and enrichment.

Lettuce samples were processed on days 0, 1, 2, 3, 6, 9, 12, 15, and 18 to enumerate the survival of *Salmonella* CFU/g stored at 4˚C or 12˚C. For each sampling day, three randomly

selected living lettuce clamshells were removed from the designated storage. The roots were removed at the base/root interface using an ethanol flamed knife and a cutting board covered with disposable cutting sheets. The knife was flame sterilized between each head and new cutting sheets used. Ten grams of root (f.w.) were transferred to a filter bag, immersed in 90 ml of 0.1% BPW, and the bag contents were homogenized for 2 minutes in a lab blender (Bag Mixer, Interscience, Weymouth, MA). Leaves were randomly removed from lettuce heads and 25 g transferred to a filter bag. Leaves were homogenized for 2 minutes in 225 ml of BPW containing 0.1% Tween 80 (Fisher Scientific) as described in *FDA's (U S Food and Drug Administration) Bacteriological Analytical Manual (BAM) (36).* Enumeration of *Salmonella* were performed using serial dilution into BPW and subsequent spread plating onto XLT-4-Rif. Numbers of *Salmonella* colonies after 48 hours of incubation at 37˚C were recorded. Additionally, 1.0 ml of homogenized contents (leaves) from stomacher bag was added to 9 ml of TSB supplemented with rifampicin and incubated at 37˚C for 24 h. After 24 h the culture was streaked for isolation on XLT-4-Rif and incubated at 37˚C for 24 h. Non-inoculated lettuce controls (n=3) were processed as above to account for contamination or growth of non-*Salmonella* bacteria on the selective media.

Statistical analysis.

Bacterial counts (CFU/g) were log transformed to approximate normal distribution. Data was subjected to one-way ANOVA using statistical software JMP® Pro 10.0 (SAS; Institute Inc., Cary, NC). The post-harvest survival of *Salmonella* log CFU/g averages recovered from two different temperatures, 4˚C and 12˚C, using 39 heads of living lettuce at each trial was statistically investigated by least significant differences (LSD), to denote differences. P-values ($P< 0.05$) with α=0.5 being considered significant.

RESULTS

Shelf life study of living lettuce.

The shelf life of living lettuce in this study was 18-days, regardless of storage temperature. After this point no further enumeration of *Salmonella* was performed.

Post-harvest transfer and survival of *Salmonella* Enteritidis on living lettuce.

On average 5.1±0.0 log CFU/g of *Salmonella* was transferred to the roots from the nutrient solution and 2.9±0.1 log CFU/g was detected on the leaves. At 4˚C and 12˚C, *Salmonella* cells persisted on the edible leaves for the 18-days of the study (Figure 4.2 and 4.3). Storage at 12˚C was associated with an increase of 1.6 log CFU/g *Salmonella* on roots of living lettuce after 18-days (P=0.0002). *Salmonella* also increased by 0.62 log CFU/g *Salmonella* on living lettuce leaves (P=0.0017) when stored at 12˚C for 18-days (Figure 4.2). No increases in log CFU/g of *Salmonella* occurred on living lettuce stored at 4˚C for 18-days (Figure 4.3). After 6 days of storage at 4˚C *Salmonella* decreased by 0.4 log CFU/g on roots (Figure 4.3), but remained unchanged throughout the remainder of the 18-day period (P<0.0001). Non-inoculated controls (n=3) yield no counts.

DISCUSSION

Post-harvest transfer of *Salmonella* and other human pathogens to produce may be associated with increased risk of transmission to humans and increased incidence of enteric disease. Post-harvest handling marks the beginning of physiological changes by cutting, trimming, and washing. During these steps post-harvest contamination spread can occur through contaminated water, via infected handler, contact surface, and knives *(5, 26)*. In this study, direct immersion of the roots in a low-nutrient solution containing *Salmonella* was used to simulate an event where the irrigation water used for hydroponic production was contaminated.

Contamination of lettuce and spinach roots has been demonstrated previously *(14, 15, 28)*. *Salmonella* serovars Typhimurium, Dublin, Cancan, Nelly, and Tamburo were able to internalize into lettuce seedlings grown in 0.5% Hoagland's agar *(14, 15)*. These studies feature inclusion of agar to the nutrient solution, which may increase interaction of motile cells with root hairs and facilitate uptake or attachment *(14, 28)*. It was beyond the scope of the current study to determine if *Salmonella enterica* were internalized through the roots and travelled through the roots to the leaf surface. Prior studies propose evidence of *Salmonella enterica* internalization *(8, 16, 29, 31)*. However, this study did recover *Salmonella* from the leaf surfaces, when only the roots were inoculated. Contamination of human pathogens from gloves and utensils have been demonstrated, with as much 2 log CFU/g from hands to lettuce leaves *(4)*. In this study, care was taken to assure that the leaves did not come into direct contact with the nutrient solution at the time of inoculation. Plastic bags were used to encase lettuce heads to prevent any transfer of *Salmonella* from handler's hands to the leaves during handling. The bags were cut from the lettuce heads after the head was placed within the clamshell to prevent further handler associated transfer. It is likely that water droplets from the roots were transferred to the plastic clamshell and then to the edible tissue during transport in and out of incubators used for storage. The interaction of pathogen survival linked to packaging material is beyond the scope of this research. As the handling steps practiced in this study were similar to those practiced by small hydroponics producers, the risk associated with post-harvest contamination of living lettuce is likely underestimated. Future studies should examine additional strategies to reduce humidity within clamshells and prevent droplet transfer.

Persistence of *Salmonella* and other human pathogens on fresh, minimally processed produce is dependent, at least in part on storage temperatures. In this study *Salmonella* persisted

at 4˚C over the 18-day shelf life. Our results indicated a decrease of 0.4 log CFU/g *Salmonella* recovered on roots after 6 days of storage at 4˚C($P<0.0001$). Our results were comparable to Hsu et al. *(12)*, first 5 days of storage at 4˚C a significantly decrease of 0.47 to 0.8 log CFU/g *Salmonella* on basil, parsley, rosemary, and cilantro was reported. Although Wu et al. *(39)* reported *Shigella sonnei* reduced by 2.5 to 3.0 log CFU/g on inoculated parsley leaves stored at 4˚C for 14 days. Tian et al. *(32)* reported no significant differences were observed in the growth of 4 pathogens on minimally processed vegetables stored at 4˚C for 15 days. It was observed *E. coli* O157:H7 and *Salmonella* Typhimurium increased by 2 log CFU/g when stored at 15˚C for 1 day. Results concluded survival and growth of pathogens were impacted by storage temperature and time *(32)*.

Pathogens on contaminated produce can grow more rapidly once chopped, releasing nutrients and water for pathogen growth held at room temperature *(26)*. Ukuku et al. *(34)* evaluated the effects of storage temperature abuse on *Salmonella* linked to fresh cut watermelon stored at 5˚C and 10˚C for 12 days. Their findings were at 5˚C *Salmonella* declined by 1 log CFU/g; yet for 10˚C *Salmonella* increased by 2 to 3 log CFU/g ($P<0.05$). This finding was consistent with our results, where the trimming of roots may have released nutrients allowing for the growth observed at 12˚C . Luo et al. *(19)* observed 2 log CFU/g statistical increase in *Escherichia coli* O157:H7 on packaged fresh-cut salads containing 'Romaine' and 'Iceberg' lettuce when held at 12˚C. Limited growth was recorded when lettuce was held at 5˚C, in agreement with our results. Oliveira et al. *(24)* report increases of 2.4 and 4.2 log CFU/g of *Salmonella enterica* and *Escherichia coli* respectively when inoculated onto shredded 'Romaine' lettuce and stored in modified atmosphere packaging (MAP) at 25˚C for 3 days. These observations are significant suggesting that both *Escherichia coli* O157:H7 and *Salmonella* can

grow, achieving amounts comparable to the average infectious dose, on packaged fresh-cut lettuce while visual quality is acceptable to consumers. This creates concerns as the potential risk of amplification of pathogens increases when living lettuce is stored at temperature abusive conditions. The interaction of pathogen survival linked to packaging material is beyond the scope of this research. Further research is necessary to conclude the survival of pathogens associated with packaging clamshells and refrigerated produce. Small scale hydroponic producers typically distribute living lettuce to farmers' markets where temperature control may be difficult to maintain below 12˚C; consumers face a significant risk in purchasing these contaminated lettuce heads. Pathogens in contaminated living lettuce have the potential to amplify and persist at temperature abusive conditions. Food safety surveys conducted in Europe, North America, and Australia indicate that consumers have knowledge gaps in refrigeration practices *(26)*. The surveys revealed up to 93% of consumers were unaware that to prevent growth of the majority of human pathogens refrigeration temperature is 0˚-5˚C *(26)*. In United States, 40-56% of the population demonstrated incorrect refrigeration temperature practices *(26)*. Results concluded from surveys conducted in Sweden that 5-20% of food items were stored at temperatures above 10˚C; majority of food items stored at temperatures ranged from 11.3-13.6 ˚C *(20)*. The largest percentage of samples (19%) held above 10˚C were Ready-To-Eat (R-T-E) salads with the maximum temperature recorded 18.2˚C *(20)*. The results of this study highlight the need to store living lettuce at 4˚C immediately after harvest to improve the microbiological safety. Refrigeration at proper temperature will prevent amplification of harmful bacteria pathogens on produce. Temperature abuse is the main source of microbial and quality deterioration in produce *(5)*. Fluctuations in temperature (<10˚C) can occur during improper storage-holding conditions, shipping and unloading of fresh-cut produce at the distributors *(5)*. Our samples were

transported on ice and maintained <10˚C in coolers to minimize fluctuations during transportation and storage to optimized shelf life quality. According to the FDA Model Food Code 2009 guidelines, produce must be received and stored at 5˚C *(2)*. This reinforces the need for maintaining temperature control to reduce bacterial pathogen contamination.

Our lettuce harvest for trial 2 study held at 12˚C had algae present on the roots. Although algae were carefully removed there was still some residue on the roots. It is unknown if the presence of algae on the roots improved or inhibited the post-harvest survival of *Salmonella*. The presence of algae can impact food safety by damaging lettuce roots or limiting valuable nutrients for growth use. Research findings on effective algae control and disinfection agents to improve living lettuce safety produced in a environmental controlled greenhouse. Further research is necessary to determine if the presence of algae improves the survival of harmful human pathogens through root attachment.

Preliminary studies were carried out to determine XLT-4 containing rifampicin, an antibiotic, as a selective agent allowing discrimination of the inoculated strain from native plant bacteria of the roots and leaves. The ideal media should have no counts when non-inoculated control roots are plated and high recovery of *Salmonella* distinctive colonies from inoculated roots. Results showed successful prevention and XLT-4-Rif selective agar plates were used for isolation of non-*typhi Salmonella* colonies for the duration of this study. Our study was performed to determine the best optimum time for soaking lettuce roots to ensure *Salmonella* recoverability. Roots were soaked in nutrient solution at variable times: 10 min, 30 min, and 60 min. There was no statistical difference in numbers of *Salmonella* recovered with the different inoculation times; therefore to duration of the studies roots were soaked in nutrient solution for 10 min.

CONCLUSION

In conclusion, the results of our study revealed that direct contamination of *Salmonella* Enteritidis on the roots of living lettuce transferred to the edible leaves; remained on roots and grew as temperature abuse conditions increased. Maintaining temperature control and risks associated with contamination in hydroponic production is emphasized by these important findings. To the best of our knowledge small scale hydroponic producers are not required by law to follow recognized safe post-harvest handling practices. A recognized food safety guideline by the FDA, "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables", has recommendations on food safety handling practices for field production of vegetable crops; however, it has not addressed food safety related to soilless vegetable crops *(37)*. Implementations of food safety guidelines coupled with risk preventions that are practical to small scale hydroponic producers are important. Application of GAPs (Good Agricultural Practices) and GHPs (Good Handling Practices) are suggested to increase food safety by minimizing pathogen contamination during post-harvest handling. This creates awareness on contamination pathways and how they may be prevented; establishing effective management practices *(2, 30, 35, 37)*. Limit resources on produce consumption without cooking to reduce illnesses are available to consumers *(2, 30, 35, 37)*. FDA recommends washing produce, removing bruised or damaged areas immediately before eating *(2, 30, 35, 37)*. Beuchat et al. *(3)* demonstrated that produce washed with potable water removes loose dirt and reduces pathogen load by 1 to 2 log CFU/g, but does not completely eliminate it. Therefore, reinforcing the importance of strict hygiene practices during harvesting and packaging is essential to maintain food safety.

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FIGURES

Figure 4.1: A series of pictures demonstrating post-harvest handling and inoculation of the heads. A) Lettuce heads and intact root systems were harvested at Virginia Tech Aquaculture Research Center Saltville, VA. B) Post-harvest Living lettuce. C) The packaged clamshells containing living lettuce with intact roots were transported on ice. D) To prevent contamination of leaves by splashing a Ziploc® plastic bag was used to encase the head with only a small hole to allow roots to protrude. E) The roots soaking in the nutrient solution containing *Salmonella* for 10 minutes. F) Wrapped roots forming a knot. G) The knot formed. H) Immediately transfer the lettuce with wrapped roots to clamshell container. I) Store the packaged lettuce in 4˚C or 12˚C, respectively, to be processed on sampling days.

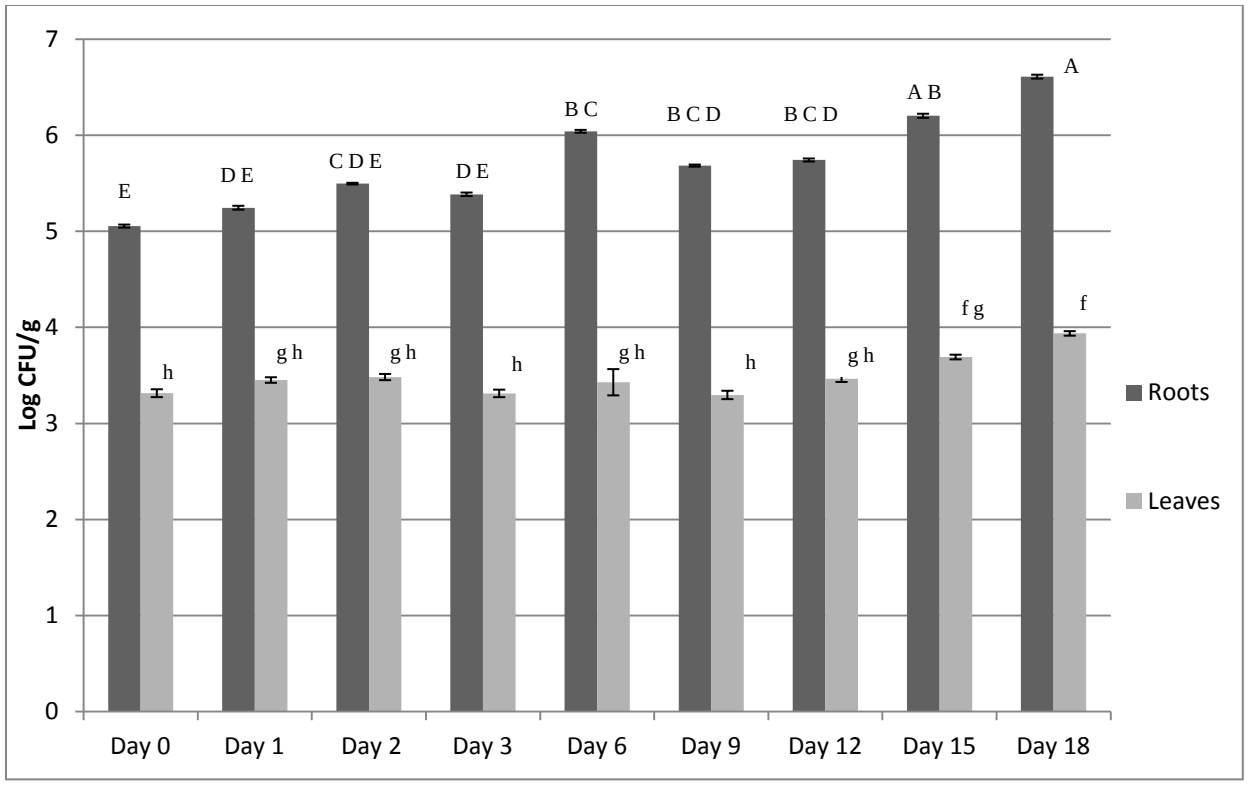

Figure 4.2. Enumeration of *Salmonella* recovered from lettuce roots and leaves of living lettuce held at 12˚C throughout the 18-day shelf life.

Bars reflect the average numbers of log CFU/g from 3 replicates destructively processed per sampling day recovered by plating on XLT4-Rif and incubated at 37˚C for 48 h. Each error bar is constructed using 1 standard deviation from the mean. Samples not connected by same letter are significantly different ($P<0.05$).

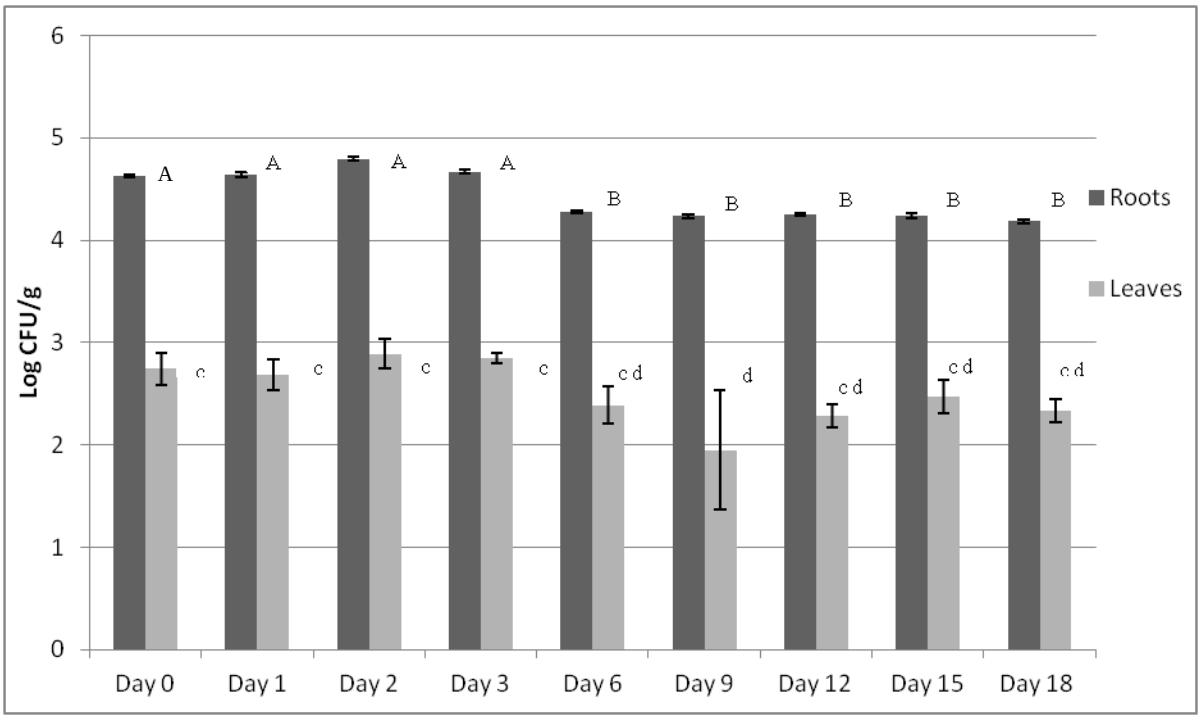

Figure 4.3: Enumeration of *Salmonella* recovered from living lettuce roots and leaves held at 4˚C throughout the 18-day shelf life.

Bars reflect the average numbers of log CFU/g from 3 replicates destructively processed per sampling day recovered by plating on XLT4-Rif and incubated at 37˚C for 48 h. Each error bar is constructed using 1 standard deviation from the mean. Samples not connected by same letter are significantly different ($P<0.05$).

CHAPTER 5: CONCLUSION AND FUTURE RESEARCH DIRECTION

The purpose of this work was to study the survival of *Salmonella enterica* serotype Enteritidis on roots and leaves of lettuce produced in a controlled greenhouse environment, packaged with intact roots in a plastic clamshell and stored at 4˚C and 12˚C for an 18-day shelf life. It was demonstrated through this study, that *Salmonella* persisted on the roots and leaves of living lettuce inoculated post-harvest when stored at 4˚C or 12˚C. This creates concerns as the potential risk of amplification of pathogens increases when living lettuce is stored at high temperatures. In addition, Glo Germ™ solution was used in the presence of *Salmonella enterica* serotype Enteritidis to demonstrate the effectiveness of current sampling strategies. This includes areas of the lettuce head most likely to be cross-contaminated during post-harvest handling of minimally processed living lettuce. Further research is necessary to explore Glo Germ™ products as a food safety training resource by promoting awareness of proper handling of leafy greens. These studies contribute to identifying post-harvest risks to ensure safer living lettuce. This research study suggests that water, and handling may be an important routes of contamination for lettuce grown hydroponically in controlled greenhouse settings: amplification of contamination and additional growth on the heads can occur during post-harvest handling, especially under temperature abusive conditions. One research question to explore further is to investigate the if the contamination risk is reduced when a large portion of the roots are removed at harvest, and does this negatively affect the shelf life. This practice may significantly reduce transfer of pathogens to edible leaves reducing contamination risks, yet maintain a good shelf life stability and premium quality. Additionally the addition of an absorbent pad to the bottom of the clamshell could reduce condensation and potentially transfer of droplets to the lettuce leaves.

Greenhouse production of vegetables is increasing in popularity, as the consumer demand for fresh, locally sourced products in increasing. It is important to expand our understanding of hydroponic production practices by small-scale producers, including post-harvest handling risks that may be applicable to field and hydroponic produced leafy greens, so interventions can be implemented at critical stages to reduce harmful pathogens. This establishes the need to implement guidelines of safer post-harvest handling practices for commercial scale hydroponic production of leafy greens. Food safety of leafy greens produced in a greenhouse hydroponic setting can be greatly improved by setting guidelines that target practices that minimize risks during post-harvest handling. In addition to practices that minimize risks, screening practices to detect contaminated leafy greens are critical. To the best of our knowledge, there is no set guideline or practice required by law regarding post-harvest handling of leafy greens in a hydroponic production. Obtaining Good Agricultural Practices (GAPs) certification is strongly encouraged to focus on the improvement of the microbiological quality of leafy greens. GAPs are critical and fundamental preventative methods to ensure microbiological safety of leafy greens by reducing human pathogen contaminations. These efforts on established practices and risk management will ensure consumers a safer leafy greens supply, which improves the public well being.