BEHAVIOR OF *ESCHERICHIA COLI* O157:H7 ON LETTUCE AND SPINACH AND *SALMONELLA* MONTEVIDEO ON TOMATOES

by

NAKIETA M. MCCULLUM

(Under the Direction of Joseph F. Frank)

ABSTRACT

Escherichia coli O157:H7 and *Salmonella* spp. are the two organisms most commonly associated with bacterial foodborne illness attributed to the consumption of fresh produce. The first study determined the behavior of *E. coli O157:H7* on the surface and after infiltration into the stem of lettuce and spinach leaves stored at 0ºC for 10 days and then shifted to 12ºC for 4 days. Statistical analyses of the results indicate greater survival of *E. coli* O157:H7 when internalized within the leaf of lettuce and spinach. The objective of the second study was to observe and compare the colonization habits and populations of *Salmonella* Montevideo, *S.* Typhimirium, and *Pseudomonas syringae* pv. tomato on the external surfaces, pulp, and seeds of flower inoculated tomato plants. One-hundred percent of *P. syringae* samples were positive, while 30% of samples were positive for *S.* Typhimirium and 80% were positive in *S.* Montevideo.

INDEX WORDS: *Salmonella*, *Escherichia coli* O157:H7, Tomato, Lettuce, Spinach

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DEDICATION

I would like to dedicate this thesis to my mother, Naomi S. McCullum. Thank you for your unwavering support. You are my rock.

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CHAPTER 1

INTRODUCTION

Escherichia coli O157:H7 and *Salmonella enterica* are the causal agents responsible for the majority of foodborne illness outbreaks, associated with the consumption of fresh and minimally processed produce *(63)*. With *E. coli* O157:H7 is commonly linked with foodborne illness among leafy green vegetables, while S. *enterica* is predominately associated with fruit, seeds, and spouts *(80)*. The increased incidences of these types of illness correlate with an increase in the consumption of fresh fruits and vegetables, and increasing populations of elderly and immune-compromised individuals. The colonization of foodborne pathogens on plants is limited by a number of factors including water and nutrient availability, exposure to UV irradiation, competition, and fluctuations in temperature and humidity.

The first study determined the survival behavior of *E. coli O157:H7* on the intact surface and after infiltration into the cut stem of lettuce and spinach leaves stored at 0ºC for 10 days and then shifted to 12ºC for 4 days. The objective of the second study was to observe and compare the colonization habits and populations of *Salmonella* Montevideo, *S.* Typhimirium, and *Pseudomonas syringae* pv. Tomato on the external surfaces, pulp, and seeds of flower inoculated tomato plants, cultivated in a growth chamber.

CHAPTER 2

LITERATURE REVIEW

Foodborne Pathogens in the Plant Phyllosphere

Although the aerial plant surface is harsh and inhospitable, it has been shown that foodborne pathogens are able to infiltrate, colonize, and survive this environment. Human pathogens face many obstacles in the aerial plant environment, including; considerable and rapid fluctuations in humidity and temperature, lack of nutrients, natural plant defense mechanisms, and competition from microorganisms better adapted to the plant phyllosphere.

The leaf surface, in particular, is a harsh environment for foodborne pathogens. Several studies have revealed that microbial populations on leaf surfaces are not spread across the leaf surface in a uniform pattern as previously suggested, but are instead localized at particular sites *(9, 54-56)*. This is most likely due to the spatial heterogeneity of available nutrients *(52)*. These studies imply that a limited number of sites on the leaf surface are advantageous for bacterial survival *(55)*.

Penetration, Attachment, and Internalization of Foodborne Pathogens on Plant Tissue

Penetration. The primary defense plants have against microbial invasion is the cuticle, a waxy outer layer that covers all parts of the plant exposed to the atmosphere *(67)*. Plant cuticles interfere with microbial colonization by limiting the passive movement of nutrients and water vapor between the plant interior and the surface *(52)*. Microorganisms penetrate the cuticle by direct penetration of the cellular wall, natural openings (e.g. stomata, hydathodes, and lenticels), or through wounds.

Attachment. Attachment to plant tissue is a vital step, in the contamination of fresh produce, for human enteric pathogens. Foodborne pathogens attach to the plant tissue rapidly and are very difficult to remove, even when treated with chlorine and/or vigorously rinsed *(61, 64, 70, 74)*. Attachment mechanisms utilized by human pathogens include polysaccharides *(34)*, bacterial lectins *(70)*, and structural adhesins (e.g., fimbra, pili, and flagella) *(14, 26)*. Several studies have demonstrated the overlapping of factors, involved in the attachment of foodborne pathogens to animal cells, and in the adhesion of plant tissue *(4-6, 45, 72)*. Barak, et.al. *(4)* demonstrated the importance of virulence genes, in the attachment of *Salmonella enterica* Newport on alfalfa sprouts. The unique mutants of *S. enterica*, selected for reduced adhesion ability, were “strains with insertions in intergenic region between agfB, the surface-exposed aggregative fimbriae (curli) nucleator, and agfD, a transcriptional regulator of the LuxR superfamily, and rpoS , the stationary-phase sigma factor *(4)*.” When compared to the wild type strain, the intergenic, rpoS, agfA, curli subunit, and agfB mutant strains, had an attachment reduction of at least one logarithmic unit. Several studies have suggested that the attachment site of foodborne pathogens on plant surfaces is affected by several factors, including plant type, strain, and environmental factors. Takeuchi et.al. *(70)* demonstrated the differential attachment of *Listeria moncytogenes*, *E. coli* O157:H7, *Salmonella* Typhimurium, and *Pseudomonas fluorescens* on lettuce surfaces. Although *Salmonella* showed no preferential attachment site, *L. monocytogenes* and *E. coli* O157:H7 favored the cut edges of the leaf, while *P. fluorescens* preferred to attach to the intact surface. Seo, et.al. *(61)*, using florescein isothicyanate labeled antibody and confocal microscopy, observed *E. coli* O157:H7 preferentially attached to the cut edges of lettuce leaves. The *E. coli* O157:H7 was also observed attached to the leaf surface, specifically in the trichomes and stomata, but in smaller numbers. It has also been observed that *E. coli* O157:H7 shows preference for attachment to lettuce roots and the deep grooves of seed coats *(75)*. Wei et.al. *(77)* studied the survival of *Salmonella* Montevideo within and on tomatoes, the results showed survival in stem scars and growth cracks after 3 days of storage.

Infiltration. Microorganisms can enter the plant apoplast through either an active or passive internalization process *(7)*. Active internalization is when the microorganism directly penetrates the cuticle or indirectly penetrates the cuticle through natural openings or wounds. Passive internalization involves the microorganisms entering the plant by means of a secondary entity (i.e. insects, aqueous solutions, aerosols, etc.) *(7)*. Several studies have demonstrated the ability of human pathogens to become internalized in plant tissue *(3, 11, 12, 46, 49, 61, 62, 68, 76, 81)*. Internalization of human pathogens have been shown to occur through natural openings *(11, 61)*, openings caused by damage *(3, 11, 31, 68)*, or through common pre-harvest/post-harvest industry practices *(49, 62)*. Once a pathogen is internalized within the plant, the organism is protected, thereby reducing the efficacy of sanitation treatments and procedures. Lettuce leaves, inoculated with an aqueous suspension of *E. coli* O157:H7, were observed with *E. coli* O157:H7 attached to the surface, trichomes, stomata, and cut edges *(61)*. The *E. coli* O157:H7, was entrapped from 20 μm to 100 μm in the stomata. Despite the use of a chlorine treatment, viable cells were found in the stomata and on the cut edges of the lettuce leaves, demonstrating the likelihood of the internalization of *E. coli* O157:H7 within the plant *(61)*. Guo, et.al*(30)*, investigated the infiltration of *S.* Montevideo, in hydroponic tomatoes. Nine days after initial root inoculation, with a nutrient solution containing *S.* Montevideo, the

Salmonella was detected in hypocotyls-cotyledons, stems, and leaves of the tomato plants *(30)*. In a similar investigation, five *Salmonella* serotypes were introduced onto tomato plants by either being brushed onto flowers or being injected into the stem. Fifty-five percent, of the Salmonella positive tomatoes, were positive for pulp contamination *(28)*. In a study by Wachtel, et.al. *(75)*, *E. coli* O157:H7 contaminated irrigation water, was used to inoculate the soil into which lettuce seedlings were planted. After several days, *E. coli* O157:H7 was quantified and was found in all parts of the plant, with very little to no population reduction *(75)*. Wounds on the plant surface allow for increased invasion by microorganisms *(31, 68)*. Han, et.al *(31)* observed *E. coli* O157:H7 on mechanically injured and un-injured green bell peppers. Although there was no initial population difference, *E. coli* O157:H7 distribution on the un-injured peppers was evenly distributed, while *E. coli* O157:H7 on the injured peppers was concentrated in the injured areas. After treatment with chlorine dioxide gas, significantly more *E. coli* O157:H7 were viable in the injured peppers compared with the un-injured vegetables *(31)*.

Factors Effecting the Colonization, Survival, and Fitness of Foodborn Pathogens within the Plant Phyllosphere

In a 1989 study, O'Brien, et.al *(58)* compared the fitness of *Pseudomonas syringae* to 18 other bacterial strains, representing five different genera , including *Escherichia coli, Salmonella* Typhimurium*, Aeromonas hydrophila,* and *Rhizobium meliloti*. Although *Pseudomonas syringae*, was significantly more fit for survival and colonization in the plant phyllosphere, the human pathogens used were also able to multiply and survive on plants. The *Salmonella*, *Escherichia*, and *Aeromonas* strains showed an increase in population on both beans and corn, even when exposed to extreme conditions, including high UV levels and low humidity *(58)*.

Brandl, et.al *(13)* compared the epiphytic fitness of *Salmonella* Thompson to *Pantoea agglomerans* and *Pseudomonas chlororaphis,* two common plant bacteria, on cilantro plants incubated at 22˚C. Although the common plant pathogens had a significantly higher population after 2 days compared to the human pathogen, the *Salmonella* serovar Thompson population increased 15-fold from the day of inoculation. Cilantro plants incubated at higher temperatures showed even high population increases for *Salmonella* Thompson *(13).* In the same study, *Salmonella* serovar Thompson displayed a drought tolerance equal to both *Pantoea agglomerans* and *Pseudomonas chlororaphis,* demonstrating *Salmonella*'s competitiveness in the plant phyllosphere *(13).*

Environmental factors, such as rain, dew, mist, and fog, induce leaching (the loss of substances/nutrients) in plants *(20).* Mature plant leaves produce substantially less leaching than younger and/or injured leaves *(20, 44).* Natural openings on the plant surface, which influence the movement of particulate matter through the plant cuticle, become more rounded as they mature *(7).* This leads to a slowdown in the respiratory process *(67)*, and decrease nutrient leaching. Brandl and Amundson *(12)* observed a 10-fold greater increase of *E. coli* O157:H7 populations on young lettuce leaves compared with older lettuce leaves. Surface analysis, of nitrogen and carbon levels, showed younger leaves were 1.5 – 2.9 times richer in both elements *(12).*

Plants, damaged by either mechanical or biological means, support enhanced epiphytic fitness. Brandl *(11)* recentlystudied the role of mechanical and biological damage in *E. coli* O157:H7 colonization of romaine lettuce. The lettuce was either mechanically bruised, cut into pieces, or infected with *Erwinia chrysanthemi* (causal agent of soft rot). Populations of *E. coli* O157:H7 on damaged leaves were 27 times greater than the population on healthy intact leaves.

Further investigation showed that *E. coli* O157:H7 colonization on the cut edges of the lettuce increased 11 fold over a period of 4 hours *(11)*. In a 10 day study, conducted by Aruscavage, et.al. *(3)*, lettuce plants damaged mechanically and biologically (with *Xanthamonas campestris*) showed enhanced survival of *E. coli* O157:H7 populations compared with the steep and quick population decline in healthy plants *(3)*.

Common plant colonizers have also been implicated in enhancing the survival and colonization of foodborne pathogens on produce. In a 1994 study, Brandl, et.al. *(15)*, discovered that a higher percentage of *Salmonella* Thompson population showed enhanced survival when enclosed in food vesicles produced by *Tetrahymena pyriformis,* a protozoa commonly found on spinach. The vesicles also provided protection against low concentrations of calcium hypochlorite *(15)*. In a further study, Gourabathini et.al.*(27)* demonstrated the ability of *Salmonella* Thompson, *E. coli* O157:H7, and *L. monocytogenes* to multiply in and exit from expelled vesicles produced by *T. pyriformis*. A study by Wells and Butterfield *(79)* showed a strong association between bacterial soft rot on fruits and vegetables and potential *Salmonella* contamination.

Risks of Pathogen Contamination During the Production of Fresh Cut Produce

Pre-harvest risks. During the pre-harvest operation, the major risks of contamination come from the use of animal waste as fertilizer and the quality of irrigation water. Recently, pre-harvest contamination of produce has become a concern, following several studies that show that *S. enterica* and *E. coli* O157:H7 are able to survive in soil, water, and manure for very long periods of time *(25, 57, 64)*. Animal manure is an ideal reservoir for foodborne pathogens. Improper treatment of animal waste, used for fertilizer, poses a significant risk of pathogen contamination in fruits and vegetables. Surveys of dairy herd manure show prevalence of *Salmonella* from 57 to 84%, and 68% in chicken waste *(66)*. Surveys of cattle feces at slaughterhouses showed 72% of samples were positive for *E. coli* O157:H7 *(66)*. There have been several studies that demonstrate the contamination of produce using manure as the inoculum source *(39, 40, 46, 57)*. A 2006 *E. coli* O157:H7 outbreak in spinach, possibly caused by fecal contaminated run-off water *(16)*, has brought attention to the potential role of water as a reservoir for foodborne microorganisms. Both *E. coli* O157:H7 and *Salmonella enterica* have been shown to infiltrate and persist in plant tissue after being introduced through contaminated irrigation water *(38-40, 65, 75)*. Potential irrigation water contamination sources, include sewage spills, run-off from animal dwelling areas, improperly treated reclaimed water, etc *(66)*.

Post-harvest risks. The post-harvest processing of fresh cut produce presents numerous risks of contamination by foodborne organisms. Worker hygiene, equipment sanitation, water quality, and temperature control are all potential risks incurred during the harvesting and processing of fresh-cut produce. Even though post-harvest industry processes expose fresh produce to numerous opportunities for contamination, very little research has been done in this area. Infiltration of fresh produce can be accelerated by increases in pressure and changes in temperature *(7)*. The cooling of produce after harvest, cause the reduction of gas pressure in the apoplast, especially if the surface openings are clogged. Until the internal temperature and the pressure difference equals the external environment, the pressure differential endures *(7)*. Li, et.al *(49)* investigated the effect of vacuum cooling, on the infiltration of *E. coli* O157:H7 in lettuce. It was shown that vacuum cooling not only significantly increased the amount of *E. coli* O157:H7 internalized within the lettuce tissue, it also enhanced survival *(49)*.

CHAPTER 3

SURVIVAL OF *ESCHERICHIA COLI* O157:H7 ON LETTUCE AND SPINACH LEAVES DIRECTLY AFTER HARVEST AS INFLUNCED BY INNOCULATION METHOD AND STORAGE TEMPERATURE[1]

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[1] McCullum, N. M. and J. F. Frank. To be submitted to *Journal of Food Protection.*

SURVIVAL OF *ESCHERICHIA COLI* O157:H7 ON LETTUCE AND SPINACH LEAVES DIRECTLY AFTER HARVEST AS INFLUNCED BY INNOCULATION METHOD AND STORAGE TEMPERATURE

Abstract

Escherichia coli O157:H7 is the causal agent most commonly identified with foodborne illness associated with leafy green vegetables. The post-harvest processing of fresh-cut produce offers many opportunities for contamination by foodborne pathogens, yet very little is known about the extent of growth and survival of internalized *E. coli* O157:H7 during post-harvest handling and processing. A five strain cocktail *E. coli* O157:H7 was spot inoculated on the intact surface and vacuum infiltrated into the cut stem of lettuce and spinach leaves. The leaves were subsequently stored at 0ºC for 10 days and then shifted to abusive conditions (12ºC) for 4 days. Statistical analyses of the results indicate greater survival of *E. coli* O157:H7 when internalized within the leaf of lettuce and spinach. The survival difference between inoculation treatments was more evident after the temperature shift to 12ºC where external populations of *E. coli* O157:H7 fell below the detection limit (2.35 log CFU/leaf) while internalized populations remained relatively constant.

Introduction

The consumption of leafy green vegetables, within the United States, increased 17.2% during 1986-1995 compared with the previous decade*(36)*. During this period, foodborne illness attributed to leafy greens increased 59.6%. Similarly, throughout the period from 1996 to 2005, consumption of leafy greens increased 9.0% and associated illness has increased by 38.6% *(36)*.

Of the foodborne illness associated with leafy green vegetables, 58.3% were caused by Norovirus, 10.4% were caused by *Salmonella*, and 8.9% were caused by *E. coli* O157:H7. Foodborne illness outbreaks associated with leafy green vegetables had a median size twice that of foodborne illness associated with other types of produce *(36)*.

The processing steps of fresh cut produce offer many opportunities for potential contamination, including harvesting, washing, cutting, cooling, packaging, and transportation. Because the processing of fresh produce does not include a viable "kill step", these products are especially vulnerable to contamination by foodborne pathogens. When foodborne pathogens become internalized in the plant tissue, this presents an especially problematical situation. Internalized pathogens are protected from surface disinfection. Previous studies have demonstrated the internalization of enteric pathogens through water uptake from the roots *(75)*, by means of surface wounds *(61, 68)*, and via natural openings on the plant surface (e.g. stomata, lenticels, and hydathodes) *(61)*.

Internalized pathogens, depending on their adaptability, are able to survive, and possibly multiply, for extended periods of time *(7)*. The growth and survival of *E. coli* O157:H7 on the surface of spinach and lettuce has been observed in previous studies *(61, 65, 70, 75)*, but little is known about the extent of growth and survival of internalized cells during post-harvest handling and processing. The objectives of this study were to determine the survival of *E. coli O157:H7* on the intact surface and after infiltration into the cut stem of lettuce and spinach leaves stored at 0ºC for 10 days and then shifted to abusive conditions (12ºC) for 4 days.

Materials and Methods

Inoculum preparation. The inoculum used in this study was prepared by mixing four strains of *E. coli* O157:H7, isolated from the feces of humans infected by consuming produce. The *E. coli* O157:H7 strains used were C7927 (apple cider), F4556 (alfalfa sprouts), H1730 (lettuce), and SEA 13B88 (un-pasteurized apple juice). The strains were grown individually at 25ºC for 16-18 h in five ml in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, Md). Prior to inoculation, the bacteria were subcultured in 10 ml of TSB and incubated at 25 ºC for 18-24 h. The bacterial cells were harvested by centrifugation (5000 x g for 5 min), washed with 0.1% peptone, re-suspended in sterile distilled water (SDW) (simulate more realistic inoculums conditions), and mixed equally to yield a four strain cocktail. The inoculum cocktail was diluted 4 µl in 2 ml SDW for spot inoculation, and 400 µl into 40 ml SDW for stem inoculation, in order to average an initial inoculum of 10^6-10^7 CFU/mL. The inocula suspensions were enumerated on tryptic soy agar (TSA).

Plant growth conditions. *Lactuca sativa* (loose-leaf lettuce, black-seeded Simpson cultivar) and *Spinacia oleracea* (spinach) were used in this study. Plants were grown in a green house specifically for this study. The benches used for plant growth were washed and sanitized using a NaOCl solution. New, 10 cm, plastic growing containers were used, and all soil remained unopened in the manufacturer's packaging, kept dry, and stored indoors until use. All seeds used, were kept vacuum sealed and stored at 42˚F, under humidity controlled conditions. All unused seed removed from the manufacturer's packaging were discarded. Before seeding, all utensils and hands were sanitized. The plants were grown in Fafard 3b, high porosity peat-lite soil, with nutrient charge (Conrad Fafard, Agawam, MA). Soil pH was maintained between 6.1 and 6.3. The plants were watered as needed using the University of Georgia water system.

Water pH was consistently 7.1 with low $CaCo_3$ and alkalinity measurements. Electrical conductivity of water was consistently below 0.01 mmhos/cm. The plants were fertilized bi-weekly using a 200 ppm concentration of "Jacks 20-10-20 General Purpose" fertilizer with Calcium and trace elements (J.P. Peters, Inc., Allentown, PA). Slow release pellets of Osmocote 14-14-14 (The Scotts Company, Marysville, OH) were applied three weeks after germination, at 14.8 g/pot. No pesticides were used on the plants during growth. The plants were grown using procedures that minimized the plants physical contact with human hands, in order to minimize bacterial contamination. Greenhouse temperature was maintained using a Wadsworth Step Controller (Wadsworth Control Systems Inc., Arvada, CO). Temperature was maintained at 22-28˚C during the day, and 17-23˚ C at night.

Harvesting of lettuce and spinach. The lettuce and spinach leaves were harvested using sterile scissors and placed in a non-sterile plastic Ziploc® bags filled with sterile tap water. The leaves were then transferred to the laboratory, immediately dipped in a chlorine solution, and placed in 5 liters of sterile tap water until inoculation. Leaves were blotted dry, using Kim® wipes before inoculation.

External inoculation of lettuce and spinach leaves. Twenty leaves of lettuce and spinach were placed, waxy side up, in a single layer, on a sterile stainless steel tray. The leaves were blotted dry and spot inoculated with 50 µl of the diluted inoculum cocktail applied to a circular area approximately 2 cm in diameter on the upper leaf surface. The inoculated leaves were then placed in Petri dishes, within the biological safety hood, and allowed to dry for 20 min.

Internal infiltration of lettuce and spinach leaves. For the internal inoculation of lettuce and spinach leaves, 20 leaves of both lettuce and spinach were cut, with a sterile scalpel, at the stem end of the leaf, to an approximate width of 19 mm. The leaves were then placed, cut end down, in 20 mL of inoculum in a 50 mL beaker, which was then placed in a vacuum chamber and exposed to an absolute pressure of 50 kPa for 20 min. Uninoculated controls were prepared for both spot inoculated and infiltrated samples.

Lettuce/spinach leaf storage. Lettuce and spinach leaves (inoculated and control) were placed into 100 mm Petri dishes (one leaf per dish). To maintain relative humidity (RH), a 70 mm #1 Whatman filter paper (Whatman Int. Ltd., Maidstone, UK) was moistened with 250 µl of SDW and secured to the top of each dish. The samples were then placed into 1 gallon Ziploc bags (10 petri dishes/bag). The atmospheric gas in the bags was replaced with a gas mixture (3% O_2, 7% CO_2, and 90% N_2) to simulate that found in lettuce retail packages. The flush gas was washed with SDW to increase the RH. The bags were flushed 9 times with a volume of storage gas four-fold larger than the volume of the Petri dishes. After every 3 flushes, the bags were held on ice for equilibration. On the ninth flush, the bags were deflated and placed into storage. The bags were reflushed every three days to maintain oxygen levels below 8% and CO_2 above 2.5%. This mixture simulated the concentration used in the fresh cut industry for lettuce, in this study the same concentration was used for spinach in order to provide a suitable comparison. The leaves were stored at 0 ºC for 10 d, and then transferred to abusive temperature conditions (12 ºC) for 4 d.

Microbiological Analysis. Aerobic bacterial plate counts (APC), and *E. coli* O157:H7 plate counts were determined for infiltrated and surface inoculated leaves and controls. *E. coli* O157:H7 was enumerated using sorbitol MacConkey agar (SMAC) and aerobic plate counts were obtained using tryptic soy agar (TSA). Samples were prepared for enumeration by blending (Oster classic beehive blender, Fisher Scientific, Pittsburgh, Penn.) the entire leaf for 30 s in 50 mL of 0.1% peptone using 236.6 mL blender jars (Ball quilted crystal jelly jars, Jarden Corp, Muncie, IN) and plating appropriate dilutions in duplicate. Prior to blending infiltrated inoculated leaves were washed with 50 mL of 0.1% peptone to remove loosely attached external cells. Samples were analyzed on days 0, 6, 10, 12, and 14.

Experimental Design. One replication consisted of 25 lettuce leaves (10 spot inoculated, 10 internal infiltration, and 5 un-inoculated controls) and 25 spinach leaves (10 spot inoculated, 10 internal infiltration, and 5 un-inoculated controls). The study was repeated three times and data was converted to log values before analysis. Analysis of variance (ANOVA) and the Duncan's procedure for the pair-wise comparison of means, for APC data and *E. coli* O157:H7 plate count data, were performed using SAS 9.1 (SAS Institute, Cary, NC).

Results

Lettuce. *E. coli* O157:H7 counts, on vacuum infiltrated lettuce leaves, trended slowly downward until day 12, but generally maintained a consistent population (Figure 2.1). An aerobic population decrease, of less than 0.5 log CFU/leaf (0.38 log CFU/leaf), in the internally inoculated lettuce, was observed by day 12, two days after the temperature shift to 12°C. This was followed by an increase in population, of almost 1 log CFU/leaf (0.94 log CFU/leaf difference) by day 14, four days after the temperature shift. Internal populations of *E. coli* O157:H7 on lettuce, showed a decrease of 0.41 log CFU/leaf by day 14. On externally inoculated lettuce leaves, aerobic populations significantly decreased by more than 1 logarithmic unit (1.17 log CFU/leaf decrease), by day 10. *E. coli* O157:H7 populations decreased by approximately 2 log CFU/leaf, during the same time period. On day 12, *E. coli* O157:H7 populations increased by 0.38 log CFU/leaf, only to resume the downward trend on day 14 by decreasing 0.93 log CFU/leaf. The aerobic populations on externally inoculated lettuce leaves decreased 1.94 log CFU/leaf over 14 days, while *E. coli* O157:H7 significantly decreased 2.59 logs CFU/leaf to below the current detection limit. The analysis of the lettuce data show significant reductions in aerobic counts for days 0, 12, and 14.

Spinach. On internally inoculated spinach leaves, aerobic populations remained relatively constant for all 14 days. Populations of *E. coli* O157:H7 showed a decrease, of 0.22 log CFU/leaf by day 10 (Figure 2.2), by day 12 (two days after the temperature shift) the population decreased by 0.55 log CFU/leaf. Overall, internal *E. coli* O157:H7 populations decreased by 0.39 log CFU/leaf over 14 days. On externally inoculated spinach leaves, aerobic and E. *coli* O157:H7 populations decreased 0.43 and 0.62 log CFU/leaf, by day 10, respectively. By day 12, after the temperature shift from 0˚C to 12˚C, *E .coli* populations decreased 1.23 log CFU/leaf. By day 14, aerobic populations on externally inoculated spinach decreased by 0.59 log CFU/leaf, while *E. coli* O157:H7 populations had decreased by almost 2.50 log CFU/leaf, to undetectable levels.

Statistical comparison of the mean *E. coli* O157:H7 counts (log CFU/Leaf), for lettuce and spinach leaves, showed significant differences between all inoculation types (internal, external, and un-inoculated) at each sampling time (Day 0, 6, 10, 12, and 14). Aerobic and *E. coli* O157:H7 plate counts, for internal inoculation of both leaf types, generally remained constant for 14 days (Figures 2.1 and 2.2). An increase in the aerobic plate counts, for internally inoculated leaves, was observed for days 12 and 14, two and four days after the temperature shift (Figures 2.1 and 2.2). On day 6, on externally inoculated lettuce and spinach, a slight decrease in *E. coli* O157:H7 and aerobic populations was observed (Figures 2.1 and 2.2). For externally inoculated spinach this trend held true on day 10, yet, for externally inoculated lettuce, a dramatic decrease was observed in both *E. coli* O157:H7 counts (4.40 to 2.90 log CFU/leaf) and aerobic counts (4.68 to 3.81 log CFU/leaf). Externally inoculated spinach demonstrated a similar decrease in population (*E. coli* O157:H7 (4.19 to 2.96 log CFU/leaf) and APC (4.40 to 4.24 log CFU/leaf) on day 12, after the temperature shift to 12˚C. By day 14, *E. coli* O157:H7 populations, for both lettuce and spinach, were nearing undetectable levels (2.35 log CFU/leaf for both lettuce and spinach). *E. coli* O157:H7 counts were significantly different, on all sampling days, for all inoculation types, in both lettuce and spinach. The population levels of *E. coli* O157:H7 in lettuce, showed larger differences in statistical means on day 10 (internal: 4.84 log CFU/leaf, external: 2.90 log CFU/leaf) compared with spinach (internal: 5.27 log CFU/leaf, external: 4.19 log CFU/leaf), this difference was eliminated after the temperature change.

Discussion

The statistical analysis point to greater survival of *E. coli* O157:H7 when internalized within the leaf of lettuce and spinach, especially at higher temperatures. This study demonstrated that external populations of *E. coli* O157:H7 decreased for both lettuce and spinach leaves, whereas, internalized populations declined yet remained relatively constant. The surface of plant leaves is considered an antagonistic environment for microbial colonization. Unfavorable conditions on plant leaf surfaces include fluctuating temperatures and relative humidity, limited and heterogeneous distribution of nutritional resources, and the waxy cuticle layer that makes attachment particularly difficult *(52)*. Potential microbial colonizers fare better if they can somehow become internalized within the plant tissue. Once internalized, microorganisms are protected from the various physiochemical fluctuations they would be exposed to on the plant surface *(7)*.

Dramatic declines in the external *E. coli* O157:H7 populations were observed on spinach leaves after day 10 and on lettuce leaves after day 6. In this study, a paper filter wetted with 1 mL of sterile distilled water was attached to the top of each Petri dish. The filter and a moistened shop towel, placed in each plastic bag containing 10 samples, were the only sources of moisture for the plant leaves. Several previous studies *(8, 9, 71)*, carried out with plant pathogenic bacteria, have demonstrated that plant leaves exposed to low levels of relative humidity had decreasing microbial surface populations while internal populations remained stable. These results correspond well with the population levels recorded in this study. The increase in the *E. coli* O157:H7 population for day 12 for external inoculation is an unusual deviation from the downward trend in microbial survival. The increase in the *E. coli* O157:H7 population at day 12 for external, lettuce, inoculation was an unusual deviation from the overall downward trend in microbial survival. The second replication of this study had an anomaly within the data, showing a large population spike of *E. coli* O157:H7, which contributed to the overall increase in population for day 12 for external inoculation in lettuce. During the second replication there was noticeable insect damage on the lettuce plants used in the study. Although leaves with conspicuous surface damage were disposed of, it is possible some of the lettuce leaves used in the study acquired surface damage that was not visible to the naked eye. Such damage could have provided additional nutrients or moisture to the surface-inoculated pathogen. This could explain the increase in *E. coli* O157:H7 survival, for external inoculation, for day 12, in the second replication. Other replications show a continued population decline, as observed on day 14 of the same replication in question. It is also worth noting, the population spike occurred two days after the temperature shift from 0°C to 12°C.

In this study a gas mixture of 3% O_2, 7% CO_2, and 90% N_2 was used in the storage of the lettuce and spinach samples. Previous studies have investigated the influence of atmospheric gas composition on the growth of *E. coli* O157:H7 in fresh-cut lettuce *(1, 19, 21, 24)*. All of the investigations used varying mixtures of O^2, N^2, and CO^2 and incorporated temperature shifts at some point during the investigation. Even at CO^2 concentrations of 30% *(21)* and atmospheres where O^2 was almost completely absent*(24)* the survival of *E. coli* O157:H7 was un-effected until the temperature shifts were incorporated. From this information, it can be concluded that temperature has more influence on the survival of *E. coli* O157:H7 than atmospheric conditions within retail packages.

A study by, Li, et.al. *(49)*, showed that infiltration by vacuum cooling significantly increased the depth of penetration and number of cells of *E. coli* O157:H7 in lettuce tissue. This study demonstrated that *E. coli* O157:H7 populations, internalized by vacuum, in lettuce or spinach tissue, can survive well within the time it takes to get to the consumer. Post-harvest contamination for produce can occur in any number of ways, including human handling, rinse water, contaminated equipment, etc. The internalization of potential foodborne pathogens in produce can be prevented by maintaining good agricultural practices (GAPs) and good manufacturing practices (GMPs).

Figures

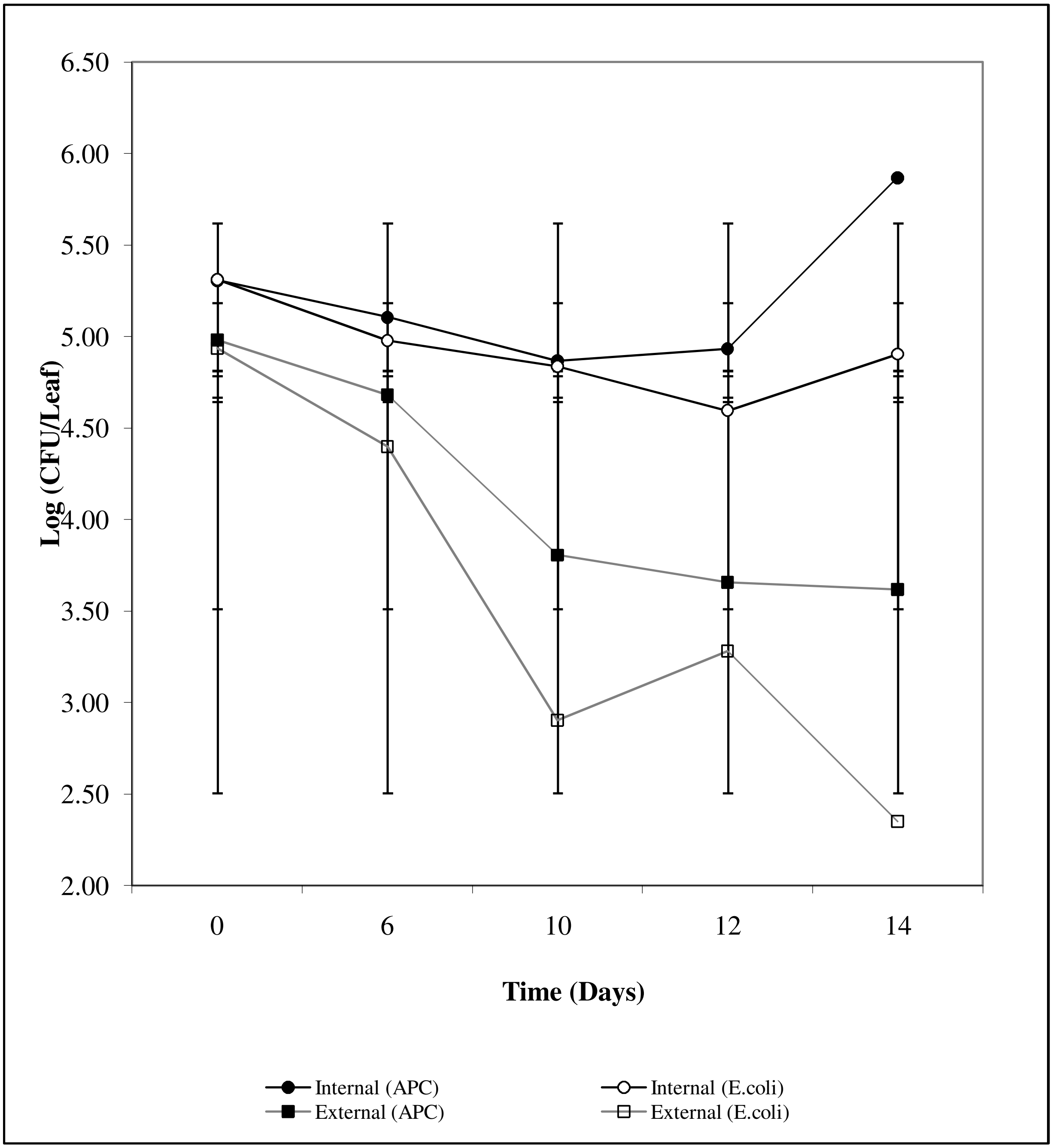

Figure 3.1 Comparison of aerobic and *E.coli* log CFU/leaf counts for internally and externally inoculated lettuce leaves. The lettuce leaves were either internally or externally inoculated with a four-strain *E.coli* O157:H7 cocktail, and then stored for 10 days at 0˚C and 4 days at 12˚C. Each data point represents the mean of log-transformed CFU values per gram of total leaf weight for 30 leaf samples (10 samples x 3 repetitions).

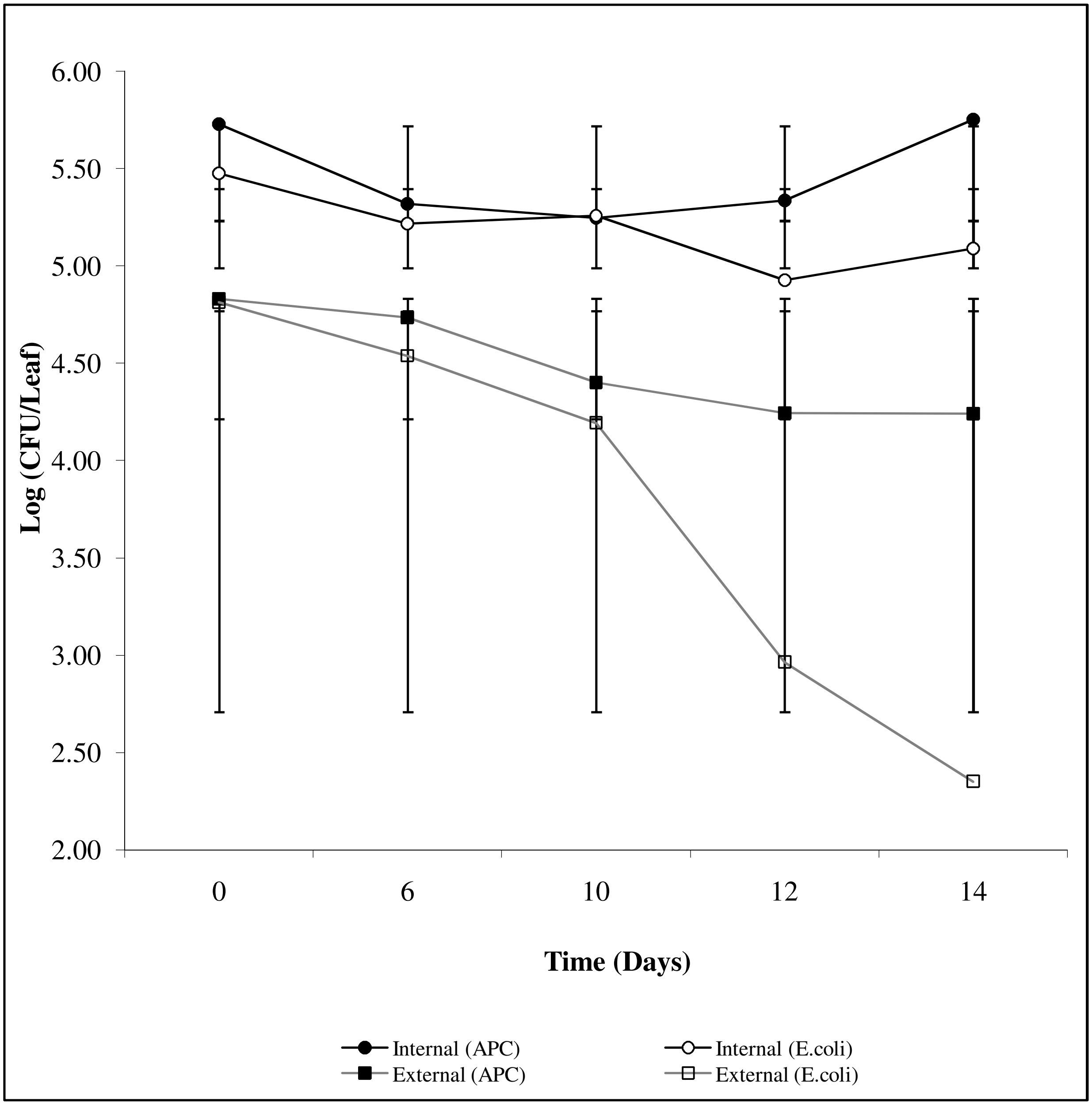

Figure 3.2 Comparison of aerobic and *E.coli* O157:H7 log CFU/leaf counts for internally and externally inoculated spinach leaves. The spinach leaves were either internally or externally inoculated with a four-strain *E.coli* O157:H7 cocktail, and then stored for 10 days at 0˚C and 4 days at 12˚C. Each data point represents the mean of log-transformed CFU values per gram of total leaf weight for 30 leaf samples (10 samples x 3 repetitions).

CHAPTER 4

COMPARISION OF THE SURVIVAL AND COLONIZATION HABITS OF *SALMONELLA* MONTEVIEDO, *S.* TYPHIMIRIUM, AND *PSEUDOMONAS SYRINGAE* PV. TOMATO ON THE SURFACE, PULP, AND SEEDS OF TOMATOES[2]

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[2] McCullum, N. M. and J. F. Frank. To be submitted to *Journal of Food Protection.*

COMPARISION OF THE SURVIVAL AND COLONIZATION HABITS OF *SALMONELLA* MONTEVIEDO, *S.* TYPHIMIRIUM, AND *PSEUDOMONAS SYRINGAE* PV. TOMATO ON THE SURFACE, PULP, AND SEEDS OF TOMATOES

Abstract

Even though tomatoes are the most common vehicle of Salmonellosis associated with fresh produce, the possibility of seed contamination has not been adequately examined. In this study the colonization habits and survival of *Salmonella* Montevideo, *S.* Typhimirium, and *Pseudomonas syringae* pv. tomato were observed on the external surfaces, pulp, and seeds of flower inoculated tomato plants, cultivated in a growth chamber. One-hundred percent of *P. syringae* samples were positive, while 30% of samples were positive for *S.* Typhimirium and 80% were positive in *S.* Montevideo. *Salmonella* Montevideo was the most prevalent in pulp samples (87.5%), but both serovars of *Salmonella* were positive in 50% of seed and external surface enrichments.

Introduction

Statistics show that Americans are becoming increasingly more nutritionally savvy. Consumption of fresh vegetables, by Americans, has increased by 23 percent, from 1970 to 2005 *(78)*. *Salmonella* accounted for 48%, of foodborne illness in the U.S., associated with fresh produce from 1973 to 1997. In 2006, there were two major *Salmonella* outbreaks, in the U.S., traced back to tomatoes. These outbreaks accounted for 23% of the total reported cases of *Salmonella* in 2006 *(35)*.

Several studies have compared the fitness of foodborne pathogens to common plant colonizers and pathogens *(13, 23, 50)*. The survival of *Salmonella* serovars in various food crops has been studied *(23, 28-30, 39, 41, 57, 59, 62, 73, 77, 81)*, yet with the exception of Arabidopsis *(18)* and alfalfa *(6, 17, 43, 50)* no analysis of attachment to seed has been conducted. The objective of this study was to observe and compare the colonization habits and survival of *Salmonella* Montevideo, *S.* Typhimirium, and *Pseudomonas syringae* pv. tomato on the external surfaces, pulp, and seeds of flower inoculated tomato plants, cultivated in a growth chamber.

Materials and Methods

Tomato plant growing conditions. The tomato variety used in this study was *Lycopersicon esculentum* (Marglobe). The seeds were sown, in a seed starting tray with the bottom covered in soil, and then topped with approximately 0.64 cm of soil. The tray was then placed in a growth chamber for approximately 7 d at 30˚C and diurnal day/night light. The seedlings were then transplanted into small trays and placed in the greenhouse with daytime temperature no warmer than 31˚C and a night temperature of no less than 13˚C. Four tomato plants were transferred to an incubation chamber (Percival Scientific Inc., Perry, IA), from the greenhouse, approximately 3 - 4 weeks after germination. Chamber humidity was maintained using two sterile containers, filled with water, placed underneath the plants. Plants were watered biweekly, and fertilized using a general fertilizer supplemented with iron and exposed to 16 h of light and temperatures of 20˚C for daytime and 25˚C at night.

Preparation of inoculum. In this study, cultures of *Salmonella* Montevideo, *S.* Typhimirium (GFP and parent strain), and *Pseudomonas syringae* pv. tomato (GFP and parent strain) were used. Strains were grown for 18-24 h in appropriate broth (Luria broth-Salmonella spp., nutrient broth-*Pseudomonas*) at 37 and 28˚C (*Pseudomonas*). The strains were subcultured, the day before inoculation, 1:10 in fresh media and allowed to incubate for 12-18 h at 37˚C. Cells were harvested by centrifugation (9500 x g for 10 min), washed with a 0.9% saline solution, and resuspended in 1 mL of peptone buffer solution (PBS). The GFP strain and parent strain of each bacterium were mixed 1:1 to yield a two strain cocktail (approximately 10^8-10^9 for *Salmonella* strains and approximately 10^4-10^5 for *Pseudomonas*). The suspensions were enumerated on nutrient agar that was incubated at 48-60 h at 25°C.

Inoculation of tomato plants. Tomato plants were inoculated at flowering stage approximately 3-4 weeks after germination. Inoculum (10 µL) was deposited on the fully open flower using a pipette. One strain of bacteria was used per tomato plant. The tomatoes were allowed to develop for 6-7 weeks and harvested at early breaker stage (approximately 60 d after germination).

Screening for *Salmonella* serovars on the tomato surface. Once harvested, the tomatoes were place into a 50 mL sterile sampling bag and 10mL of buffered peptone water (BPW) was added. The tomato was massaged by hand for 2 min and 350 µL of the suspension was spread plated onto xylose lysine desoxycholate (XLD) agar, Luria-Bertani (LB) agar, and Hektoen enteric (HE) agar, both plain and supplemented with 100 µg/mL of ampicillin (for GFP strain), in duplicate. The plates were incubated for 18-24 h at 37°C and enumerated.

Screening for *Pseudomonas syringae* pv. tomato on the tomato surface. Tomatoes were screened for *Pseudomonas* same as above with the exception of type of media used. Phosphate buffer solution (PBS) was used in the place of BPW. For *Pseudomonas*, 250 µL of an appropriate dilution was plated on nutrient agar (NA) supplemented with 100 µg/mL of rifampicin and/or 25 µg/mL of kanamycin. The plates were incubated for 48-60 hours at 25°C and then enumerated.

Screening for *Salmonella* serovars in tomato pulp. Tomatoes were washed in tap water until visibly clean and then dipped in a 2% sodium hypochlorite solution for 10 min. The tomatoes were removed and dipped in sterile distilled water three times and patted dry. Using a sterile scalpel, the pericarp was removed, and the ovary tissue was into quarters. Each quarter was placed into a sterile sample bag with 10 ml of sterile buffered peptone water (BPW) and stomached for 90 s. The suspension was mixed by swirling and pH was adjusted with hydrochloric acid (HCL) to 6.8 ± 0.2. Three hundred and fifty microliters (350µL) of the suspension was spread plated onto XLD, LB, and HE agar, both plain and supplemented with 100 µg/mL of Ampicillin (for GFP strain), in duplicate. The plates were incubated for 18-24 hrs at 37°C and enumerated.

Screening for *Pseudomonas* in the tomato pulp. Tomatoes were screened for *Pseudomonas* same as above with the exception of type of media used. Phosphate buffer solution (PBS) was used in the place of BPW. For *Pseudomonas*, 250 µL of an appropriate dilution was plated on NA supplemented with 100 µg/mL of rifampicin and/or 25 µg/mL of kanamycin. The plates were incubated for 48-60 h at 25°C and then enumerated.

Enrichment of tomato samples (*Salmonella*). The remaining liquid samples were incubated for 18-24 h at 37°C and transferred 1:10 ml to tetrathionate (TT) broth. The enrichment was incubated for 18-24 h at 37°C and streaked onto xylose lysine desoxycholate (XLD) agar, Luria-Bertani (LB) agar, and Hektoen enteric (HE) agar, both plain and supplemented with the appropriate antibiotics, in duplicate. The streaks were incubated for 18-24 h at 37°C and then screened for positives. Samples positive for *Salmonella* were confirmed using microscopy (GFP strain) or using *Salmonella* Chromagar®.

Screening for *Salmonella* serovars in tomato seeds The seeds were removed using sterile forceps, and placed in a sterile stainless steel container, lined with absorbent material. The seeds were allowed to dry for 24 h under a biosafety hood. The seeds were then weighed, and placed in a sterile ceramic mortar containing 1 mL of BPW. The seeds were then crushed until completely macerated. The paste was then removed and placed into a sterile sample bag containing 9 ml of BPW. Three hundred and fifty microliters (350µL) of the suspension was spread plated onto XLD, LB, and HE agar, both plain and supplemented with the appropriate antibiotics (for GFP strain), in duplicate.

Enrichment of tomato seeds (*Salmonella*). The remaining suspensions were incubated for 18-24 hrs at 37°C, transferred 1:10 ml to BPW, and then incubated for 48 hrs at 37°C. The enrichment was then transferred 1:10 ml to tetrathionate (TT) broth, incubated again for 18-24 h at 37°C, and streaked onto XLD, LB, and HE agar, both plain and supplemented with the appropriate antibiotics, in duplicate. The streaks were incubated for 18-24 h at 37°C and then screened for positives. Presumptive positives for *Salmonella* were confirmed using microscopy (GFP strain) or by streaking positive samples onto *Salmonella* Chromagar ® and incubating for 18-24 h at 37°C.

Screening for *Pseudomonas* on tomato seeds. Tomatoes were screened for *Pseudomonas* same as above with the exception of type of media used. Phosphate buffer solution (PBS) was used in the place of BPW. For *Pseudomona*s, 250 µL of an appropriate dilution was plated on NA supplemented with 100 µg/mL of rifampicin. The plates were incubated for 48-60 h at 25°C and then enumerated.

Results

The tomato plants produced a total of seven tomatoes, two inoculated with *Salmonella* Montevideo, two inoculated with *S.* Typhimirium, two inoculated with *Pseudomonas syringae* pv. tomato, and one uninoculated control. All uninoculated samples were negative for *Salmonella* Montevideo, *S.* Typhimirium, and *Pseudomonas syringae* pv. tomato.

Pseudomonas syringae was introduced onto tomato flowers at an initial inoculum of 10^5-10^6 CFU/mL. All of the *Pseudomonas* enrichments were positive (Table 3.1). *P. syringae* surface samples showed average counts of approximately 10^3 CFU/mL, while pulp plate counts averaged 10^3-10^5 CFU/g (Table 3.2). *Salmonella* Typhimirium had an average initial inoculum count of 10^8 CFU/mL. Analysis of samples revealed, surface and pulp, *S. typhimirium* populations of less than 10^2 CFU/mL(g) (Table 3.3). Thirty percent of all samples were positive for *S.* Typhimirium (Table 3.1). Of the surface, pulp, and seed samples 50%, 25%, and 50%, respectively, were positive for *S.* Typhimirium (Table 3.1). The average initial inoculum of *Salmonella* Montevideo was 10^8-10^9 CFU/mL. The average surface counts were less than 10^2 CFU/mL, while pulp counts ranged from less than 10^2 CFU/g to 10^3 CFU/g (Table 3.4). Eighty percent of all enrichment samples were positive for *S.* Montevideo(Table 3.1). *Salmonella* Montevideo was isolated from 50% of surface samples, 87.5% of pulp samples, and 50% of seed samples (Table 3.1).

Discussion

In a 1989 study, O’Brien, et.al *(58)* compared the fitness of *Pseudomonas syringae* to 18 other bacterial strains, representing five different genera , including *E. coli, S.* Typhimurium*,* *Aeromonas hydrophila,* and *Rhizobium meliloti.* Like, the O'Brien study, the current study demonstrates the superior fitness of and *Pseudomonas syringae* pv. tomato, a common plant pathogen of tomatoes, compared with that of the enteric bacteria. Unlike *P. syringae*, the *Salmonella* serovars are not equipped with the ability to utilize the majority of nutrients available in produce *(10)*. The inability to utilize sucrose (one of the main sugars produced by plants) limits the colonization ability of *S. enterica* (and other enteric bacteria) on and within plants*(10)*.

Guo and others *(28)*, studied the survival of five *Salmonella* serotypes in tomatoes, produced by plants inoculated by stem injection or by brushing inoculum onto the flowers. Of the five serovars, *S.* Montevideo displayed the most persistence, surviving 49 days after inoculation and being present on/in the most tomatoes (36%) *(28)*. In this study, the *Salmonella* serovars survived for 45-56 d, from time of inoculation to the time of harvest. Shi, et.al. *(62)* compared the fitness of several *Salmonella* serovars, consisting of strains isolated from both animal and outbreak (associated with produce) sources. The inoculum was introduced onto the flowers of tomato plants and the subsequent tomatoes were harvested at the breaker stage. *Salmonella* Montevideo, isolated from tomato, was positive in 90% of the internal samples and 40% of the external samples. In contrast, *S.* Typhimirium, isolated from a porcine source, was not found in any of the internal samples and positive in 38% of external samples *(62)*. Similarly, in the current study, *S.* Montevideo was found in 87.5% of pulp enrichments compared to 25% for *S.* Typhimirium. Although, the number of positive surface and seed enrichments were the same for both serovars, the lack of samples tested could account for this.

This study demonstrated the possibility of *Salmonella* attachment on the seeds of tomatoes, regardless of the serovars fitness in other parts of the tomato. Seeds are considered an important source of inoculum in the contamination cycle of many plant-pathogenic microorganisms*(2)*, yet there has been very little research on seeds as an inoculum source for foodborne pathogens. Cooley, et.al *(18)* observed the colonization of *S. enterica* and *E. coli* O157:H7 on Arabidopsis thaliana, grown in contaminated soil. *Salmonella enterica* and *E. coli* O157:H7 was observed throughout the plant, including the seeds, roots, eternal surfaces, and flowers. Liao and Fett*(51)*, showed that *Salmonella* was able to survive on alfalfa seeds for approximately 2 -3 years in storage*(51)*. The presence of *Salmonella* on alfalfa seeds*(22, 50)*, and the subsequent survival after the planting and germination has been observed in several studies, but this has yet to be observed for other potential plant reservoirs.

Tables

Table 4.1 Predominance of *Salmonella* Typhimirium, *S.* Montevideo, and *Pseudomonas syringae* on and within fruit and seeds resulting from inoculated tomato flowers.

		Surface	Pulp	Seeds
	No.of samples	Number (percent) of positive enrichments		
Salmonella Typhimirium	12	1 (50)	2 (25)	1 (50)
Salmonella Montevideo	12	1 (50)	7 (87.5)	1 (50)*
Pseudomonas syringae	12	2 (100)	8 (100)	2 (100)

*Although both samples produced black colonies on the XLD and HE plates, only one was confirmed as *Salmonella*.

Table 4.2 Mean CFU/g (mL) counts of *Pseudomonas syringae pv.tomato* on the skin, pulp, and seeds of flower inoculated tomatoes grown in an incubation unit.

	Nutrient Agar	Nutrient Agar (w/ 100 µg/mL Rifampicin)	Nutrient Agar (100 µg/mL Rifampicin and 25 µg/mL Kanamycin)	Enrichment
	CFU/g (CFU/mL)			
External 1	2.38E+03	2.50E+02	<2.50E+02	+
Pulp 1.1	5.12E+02	3.33E+02	3.57E+02	+
Pulp 1.2	2.60E+03	1.40E+03	1.55E+03	+
Pulp 1.3	2.59E+03	8.49E+02	9.43E+02	+
Pulp 1.4	1.82E+03	1.04E+03	1.86E+03	+
Seed 1	1.35E+04	1.04E+04	<2.50E+02	+
External 2	4.63E+03	3.50E+03	<2.50E+02	+
Pulp 2.1	2.78E+03	1.59E+03	1.59E+03	+
Pulp 2.2	1.04E+03	9.18E+02	1.15E+03	+
Pulp 2.3	1.39E+03	2.31E+03	1.30E+03	+
Pulp 2.4	7.79E+02	5.25E+02	7.07E+02	+
Seed 2	1.25E+04	<2.50E+02	<2.50E+02	+

Table 4.3 Mean CFU/g (mL) counts of *Salmonella* Typhimirium on the skin, pulp, and seeds of flower inoculated tomatoes grown in an incubation unit.

	LB Agar	**LB Agar** (w/ 100µg/mL Ampicillin)	**XLD Agar**	**XLD Agar** (w/ 100µg/mL Ampicillin)	**HE Agar**	**HE Agar** (w/ 100µg/mL Ampicillin)	**Enrichment**
	CFU/g (CFU/ml)						
External 1	2.17E+02	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Pulp 1.1	4.07E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Pulp 1.2	3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Pulp 1.3	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Pulp 1.4	3.30E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Seed 1	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
External 2	6.65E+01	2.45E+01	1.40E+01	5.25E+00	2.98E+01	5.25E+00	+
Pulp 2.1	3.40E+01	1.98E+02	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	+
Pulp 2.2	3.97E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Pulp 2.3	1.68E+01	1.05E+02	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	+
Pulp 2.4	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	-
Seed 2	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	< 3.08E+00	+

Table 4.4 Mean CFU/g (mL) counts of *Salmonella* Montevideo on the skin, pulp, and seeds of flower inoculated tomatoes grown in an incubation unit.

	LB Agar	**XLD Agar**	**HE Agar**	**Enrichment**
S. Montevideo	**CFU/g (CFU/ml)**			
External 1	3.66E+00	< 3.08E+00	< 3.08E+00	-
Pulp 1.1	1.63E+01	< 3.08E+00	< 3.08E+00	-
Pulp 1.2	4.87E+01	< 3.08E+00	< 3.08E+00	-
Pulp 1.3	1.85E+01	< 3.08E+00	< 3.08E+00	+
Pulp 1.4	< 3.08E+00	< 3.08E+00	< 3.08E+00	+
Seed 1	< 3.08E+00	< 3.08E+00	< 3.08E+00	-*
External 2	2.03E+02	1.37E+02	1.75E+02	+
Pulp 2.1	8.44E+01	< 3.08E+00	2.53E+01	+
Pulp 2.2	1.07E+01	< 3.08E+00	< 3.08E+00	+
Pulp 2.3	1.36E+01	< 3.08E+00	< 3.08E+00	+
Pulp 2.4	< 3.08E+00	< 3.08E+00	< 3.08E+00	+
Seed 2	< 3.08E+00	< 3.08E+00	< 3.08E+00	+

CHAPTER 5

SUMMARY AND CONCLUSIONS

Outbreaks of foodborne illness, associated with fresh produce, increased from less than 1% of overall foodborne illness in the 1970 to 6% in the 1990s *(53)*. In the current studies, emphasis was placed on the survival of two species of bacterial foodborne pathogens, most commonly associated with the contamination of fresh produce.

The first study focused on survival rates of *E. coli* O157:H7 on the surface and after infiltration into the cut stem of lettuce and spinach leaves which were subsequently stored at 0ºC for 10 d and 12ºC for 4 d. The study demonstrated greater survival of internalized populations of *E. coli* O157:H7 compared with populations on the leaf surface even at proper cold storage and retail temperatures/conditions. *E.coli* O157:H7 populations on the surface of both lettuce and spinach showed a sharp decline after day 6, while internalized populations remained relatively constant for all 14 d. This finding is supported by several similar studies on the fitness of foodborne pathogens on and within plant tissue *(1, 3, 11, 12, 18, 25, 31-34, 37, 38, 42, 46-49, 60, 61, 65, 68-70, 72, 75, 76)*.

The second study compared the fitness of *Pseudomonas syringae* pv. tomato, *Salmonella* Typhimirium, and *S.* Montevideo on/in the surface, pulp, and seeds of tomatoes derived from incubator grown, flower inoculated tomato plants. *Pseudomonas syringae* pv. tomato, a common pathogen of tomato plants, was isolated from 100% of surface, pulp, and seed samples, demonstrating superior fitness compared with the *Salmonella* serovars. Of the *Salmonella* serovars, *S.* Montevideo was isolated from 87.5% of all samples compared to 33% of positive *S.* Typhimirium samples. Although *Salmonella* Montevideo was isolated from a greater percentage of pulp samples, the analyses showed no difference in survival of either *Salmonella* serovars on surface or seed samples. It is unclear whether this finding is significant due to the small number of samples analyzed and the lack of previous research on *Salmonella* survival on tomato seeds.

Despite the increase in awareness, large gaps in the knowledge of enteric pathogen-plant interaction, deficiencies in regulatory oversight, and industry negligence in developing adequate GMPs/GAPs, the incidence of these types of illnesses are likely to continue to increase, as the demand for fresh produce also increases.

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APPENDIX

APPENDIX

Analysis of variance of data (ANOVA) for survival of *E. coli* O157:H7 (sorbitol McConkey agar counts) on spinach leaves with internal (stem) and external (spot) inoculation.

Source	DF	F-value	Pr>F
Model	14	130.35	<.0001
Error	60		
Trt	2	826.80	<.0001
Time	4	22.23	<.0001
Time*Trt	8	10.30	<.0001

Analysis of variance of data (ANOVA) for total aerobic counts on spinach leaves with internal (stem) and external (spot) inoculation.

Source	DF	F-value	Pr>F
Model	14	6.42	<.0001
Error	60		
Trt	2	40.37	<.0001
Time	4	0.89	0.4741
Time*Trt	8	0.89	0.6973

Analysis of variance of data (ANOVA) for survival of *E. coli* O157:H7 (sorbitol McConkey agar counts) on lettuce leaves with internal (stem) and external (spot) inoculation.

Source	DF	F-value	Pr>F
Model	14	117.64	<.0001
Error	60		
Trt	2	735.92	<.0001
Time	4	22.92	<.0001
Time*Trt	8	10.43	<.0001

Analysis of variance of data (ANOVA) for total aerobic counts on lettuce leaves with internal (stem) and external (spot) inoculation.

Source	DF	F-value	Pr>F
Model	14	17.05	<.0001
Error	60		
Trt	2	103.13	<.0001
Time	4	2.08	0.0943
Time*Trt	8	3.27	0.0037