

**USING LABORATORY- AND FIELD-BASED TRIALS TO UNDERSTAND
ENVIRONMENTAL MICROBIAL FOOD SAFETY ISSUES ASSOCIATED
WITH RAW AGRICULTURAL COMMODITIES**

by

Patrick Michael Spaninger

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

Fall 2017

© 2017 Patrick Michael Spaninger
All Rights Reserved

**USING LABORATORY- AND FIELD-BASED TRIALS TO UNDERSTAND
ENVIRONMENTAL MICROBIAL FOOD SAFETY ISSUES
ASSOCIATED WITH RAW AGRICULTURAL COMMODITIES**

by

Patrick Michael Spaninger

Approved: _____
Limin Kung Jr., Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____
Mark Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Ann. L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Kalmia E. Kniel, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Dallas G. Hoover, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Gordon Johnson, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Manan Sharma, Ph.D.
Member of dissertation committee

ACKNOWLEDGMENTS

I would like to thank Dr. Kali Kniel, my mentor and adviser. She took a chance on me, for that I will forever be grateful. She provided me with the tools to succeed, and asked only for my effort and dedication in return. Even when I did not believe in myself and she has always had my back. She allowed me to make my own mistakes in order to fully understand and appreciate the process of scientific method and research. She supported my scientific interests and passions in the laboratory, allowing me to become a confident and well-rounded scientist. There are not enough words to express how much I adore and appreciate this woman for all she has done for me. For all these reasons, I will forever be indebted to her.

I would also like to thank all of the members of the Kniel lab, past and present. I want to express my deepest heartfelt gratitude to Qing Wang, Sarah Markland, Kyle LeStrange, Kirsten Hirneisen and Adrienne Shearer who helped provide guidance especially at the beginning of my graduate career when I was completely new to research. I would also like to thank, Thais Del Ramos, Shani Craighead, and Angela Ferelli for their friendship and for bringing a sense of humor to the lab every single day. I would also like to thank, Ro Steiner, June Teichman, Amanda Chasten, and, Claire Marik for all their efforts and assistance in the laboratory, especially dealing with me every day.

I would also like to especially thank Dr. Gordon Johnson for helping me to become a vegetable grower. I would also like to thank Dr. Dallas Hoover and Dr. Manan Sharma for their guidance over these last five years as mentors and committee

members. I feel honored and humbled to have had the opportunity to work with and learn from two amazingly talented food scientists.

Lastly, I have to thank my family for believing in me. Their love and support has guided me through this process. To my mom and dad, thank you for being proud of me no matter the circumstances and providing me with every opportunity to reach my goals. To Molly, you have my heart and you are my motivation. Thank you for always striving for perfection and for loving me. To Wilson, thank you for being so darn cute and I am so proud to call you my son. Each day you grow bigger in size and in my heart. My hope is that I can provide you the ability to chase your dreams every day. To Colleen, Monica, Matthew, Shane, and Siobhan, thank you for being my oldest friends and for being patient with me even in difficult times. To Jeff, Judy, Sean, Barbara, Beverly, Noah, Emily, Justin, and Megan, thank you for being the best in-laws and role models to the next generation of our family (Brigid, Cienna, Grace, Jackson, Nellie, Theo, Rowan, Nash, Yarrow and Baby Green)

This dissertation is just the beginning. While in graduate school, I have matured. These last five years of my life, have challenged me in so many ways and helped me to learn so many things. It takes blood, sweat, and tears to achieve your goals. My time at Delaware has been one of the most exciting periods of my life. I will never forget all the staff, friends, family, and advisors who supported my endeavors along the way.

This dissertation is dedicated to my Grandparents

TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	x
ABSTRACT	xiii

Chapter

1	RESEARCHERS' GUIDE: PRE-HARVEST ENVIRONMENTAL MICROBIAL FOOD SAFETY ISSUES ASSOCIATED WITH RAW AGRICULTURAL COMMODITIES	1
1.1	Introduction	1
1.2	Produce Outbreaks (2012-2016).....	3
1.3	Routes of Pre-Harvest Contamination.....	5
1.4	Agricultural Water.....	5
1.5	Animal intrusion.....	8
1.6	Worker Health and Hygiene.....	9
1.7	Soil Amendments	11
1.8	Implications of the Produce Rule: Guidelines for Pre-Harvest Regulations.....	12
1.9	Weather and Food Safety	14
	REFERENCES	16
2	USE OF GENERIC <i>E. COLI</i> AS AN INDICATOR OF POOR MICROBIOLOGICAL WATER QUALITY IN IRRIGATION WATER AND ON FRESH FRUITIS AND VEGETABLES PRIOR TO HARVEST. ..	31
2.1	Abstract.....	31
2.2	Introduction	32
2.3	Materials and Methods	35
2.3.1	Preparation of Manure Inoculum.....	35
2.3.2	Plant Preparation.	36
2.3.3	Plant Inoculation.....	37
2.3.4	Sampling and <i>E. coli</i> Enumeration.....	37
2.3.5	Confirmation of <i>E. coli</i> Isolates.....	38

2.3.6	Quanti-Tray Colilert Enumeration	38
2.3.7	Weather Data	38
2.3.8	Statistical Analysis	38
2.4	Results	39
2.4.1	Tomato Field Trials	39
2.4.2	Leafy Green Field Trials	41
2.5	Discussion.....	44
	REFERENCES	55
3	USE OF ZERO-VALENT IRON FILTRATION TO ENHANCE IRRIGATION WATER QUALITY	62
3.1	Abstract.....	62
3.2	Introduction	63
3.3	Materials and Methods	66
3.3.1	Preparation of Manure Water Contaminated with <i>E.coli</i>	66
3.3.2	Salmonella Culture Preparation for Inoculated Pond Water.	67
3.3.3	Filter Preparation	68
3.3.4	Filtration of Water Samples.....	68
3.3.5	Plant Preparation	70
3.3.6	Plant Inoculation.....	71
3.3.7	Sampling and <i>E. coli</i> Enumeration	71
3.3.8	Column Deconstruction.....	72
3.3.9	Water Analysis	72
3.4	Results and Discussion	73
	REFERENCES	80
4	THE FATE OF SIX <i>SAMONELLA</i> AND FIVE <i>E. COLI</i> SEROTYPES IN DIFFERENT WILD ANIMAL FECES OVER A YEAR	86
4.1	Abstract.....	86
4.2	Introduction	87
4.3	Materials and Methods	91
4.3.1	Serotypes Cocktail Profile.....	91
4.3.2	Inoculum Preparation	91
4.3.3	Cattle and Wild Animal Fecal Preparation.....	92

4.3.4	Cocktail Inoculation and Bacterial Enumeration	93
4.3.5	STEC and Salmonella Enrichments	93
4.3.6	Typing Banked Isolates and Pulse-Field Gel Electrophoresis (PFGE).....	94
4.3.7	Statistical Analysis	95
4.4	Results and Discussion	95
	REFERENCES	107
5	OBERSVATIONS OF <i>E. COLI</i> ISOLATES IN RESPONSE TO SIMULATED GASTRIC FLUID	115
5.1	Abstract.....	115
5.2	Introduction	115
5.3	Materials and Methods	119
5.3.1	Preparation of Manure Water Contaminated with <i>E.coli</i>	119
5.3.2	Preparation of Bacterial Inoculums	119
5.3.3	Preparation of Simulated Gastric Fluid	120
5.3.4	Spinach Inoculation	120
5.3.5	Simulated Gastric Fluid Assay	121
5.3.6	PCR Analysis.....	122
5.3.7	Statistical Analysis	122
5.4	Results	123
5.5	Discussion.....	125
	REFERENCES	133

LIST OF TABLES

Table 1. <i>E. coli</i> positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2014.	46
Table 2. <i>E. coli</i> positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2013.	46
Table 3 <i>E. coli</i> positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2012.	47
Table 4. Populations of generic <i>E. coli</i> and <i>S. Newport</i> in filtered water.	79
Table 5. The average <i>Salmonella</i> population and standard deviation at each sampling point for five fecal types sourced in the Mid-Atlantic United States.	102
Table 6. The average STEC population and standard deviation at each sampling point for five fecal types sourced in the Mid-Atlantic United States.	103
Table 7: The rate of reduction and R ² values for <i>E. coli</i> and <i>Salmonella</i> on five fecal types sourced in the Mid-Atlantic United States.	104
Table 8. Multiplex Assays for the screening of Eight EHEC virulence genes.	129
Table 9. Distribution of EHEC genes, source and sample day for <i>E. coli</i> isolates.	129

LIST OF FIGURES

Figure 1. E. coli concentrations from tomatoes harvested and enumerated on TBX in Delaware from 2012 to 2013.....	47
Figure 2. E. coli concentrations from lettuce plants harvested and enumerated on TBX in Delaware from 2013 to 2014.....	48
Figure 3. E. coli concentrations from spinach plants harvested and enumerated on TBX in Delaware from 2013 to 2014.....	49
Figure 4. The average air temperature (solid line) and radiation (dashed line) from 31 August through 10 September 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with E. coli.	50
Figure 5. The total rainfall (solid line) and average wind velocity (dashed line) from 31 August through 10 September 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with E. coli.....	51
Figure 6. The average air temperature (solid line) and radiation (dashed line) from 12 August through 23 August 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with E. coli.	51
Figure 7. The total rainfall (solid line) and average wind velocity (dashed line) from 12 August through 23 August 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with E. coli.....	52
Figure 8. The average air temperature (solid line) and radiation (dashed line) from 31 May through 10 June 2013 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with E. coli.	52

Figure 9. The total rainfall (solid line) and average wind velocity (dashed line) from 31 May through 10 June 2013 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with <i>E. coli</i>	53
Figure 10. The average air temperature (solid line) and radiation (dashed line) from 4 June through 14 June 2014 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with <i>E. coli</i>	53
Figure 11. The total rainfall (solid line) and average wind velocity (dashed line) from 4 June through 14 June 2014 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with <i>E. coli</i>	54
Figure 12. Experimental layout and dimensions for 12 plots containing tomato plants.	54
Figure 13. Generic <i>E. coli</i> recovery (log CFU ml ⁻¹) within either sand or sand-ZVI filter column following treatment of manure water. Deconstructed columns were sectioned in four equal (TOP, MID, MID2, BOT) regions measuring 0.125 m. Within whole model, daily means across both filters followed by different letters (a,b,c,d,e,f,g) are significantly different (P<0.05). Super fine sand was placed in TOP section, while MID and MID2 contained fine sand. The BOT section was composed on level two media which was slight larger in grain size than both upper regions.	78
Figure 14. Appearance and characteristics for fecal types sourced in the Mid-Atlantic United States.....	105
Figure 15. Populations of STEC on feces over one year period.	105
Figure 16. Populations of Salmonella on feces over one year period.	106
Figure 17. Flowchart depicting the procedure for culturing <i>E. coli</i> isolates and inoculation of spinach and SGF.	127
Figure 18. Calculated percent survival for <i>E. coli</i> isolates after 2 hours (top) and 5 hours (bottom) exposure to simulated gastric fluid.....	128

- Figure 19. Persistence of laboratory and wild-type EHEC following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an * 130
- Figure 20. E. coli persistence following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an * 131
- Figure 21. E. coli persistence following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an * 132

ABSTRACT

This document outlines pre-harvest food safety challenges, recent U.S. multistate outbreaks, and routes of contamination linked to fresh produce. There are numerous field trials and laboratory studies that will be presented, producing a strong foundation for effective environmental food safety research. In the sections to follow, a review of production practices will be discussed. Soil amendments, irrigation, and harvesting may increase the potential for foodborne outbreaks including bacterial pathogen survival and transmission to water, soil and plant milieu. This project attempts to encompass all of these issues into one document to further important understand factors involved in cross-contamination of produce in production areas. In turn, this project hopes to generate a guidebook to reduce the burden of foodborne illness associated with fruits and vegetables.

Chapter 1

RESEARCHERS' GUIDE: PRE-HARVEST ENVIRONMENTAL MICROBIAL FOOD SAFETY ISSUES ASSOCIATED WITH RAW AGRICULTURAL COMMODITIES

1.1 Introduction

Foodborne outbreaks as a result of environmental microbial contamination have growing public health implications and financial costs. The projected total economic cost due to produce-associated foodborne illnesses is estimated to be more than \$70 billion (Scharff, 2012). A high profile example, often referred to as a turning point in the produce industry occurred in September 2006, when a *E. coli* O157:H7 outbreak linked to bagged spinach rocked the spinach industry, resulted in a loss of \$200-300 million of recalled product and consumer uncertainties (Warriner *et al.* 2009). In total, the outbreak reached 27 states causing 116 hospitalizations and 5 fatalities (Sharapov *et al.* 2016). While the outbreak was still ongoing the FDA announcement warning not to eat spinach was released (Arnade *et al.* 2010). In my opinion, the rise of social media outlets has played a role in the sharing of information to a wider audience. Prior to this outbreak, rarely did a regulatory body get involved while the outbreak was active (Arnade *et al.* 2010).

Produce outbreaks have been on the rise since 1990 (CSPI, 2008). Between 1990 and 1998, the number of global produce outbreaks was as low as 5 in 1992 to 45 in 1998. Skyrocketing in 1999, annual produce outbreaks ranged from 55-80 per year from 1999-2006 (CSPI, 2008). In fact, from 1998 to 2008, contaminated produce was associated with 23% of U.S. reported foodborne illnesses (Klein and Smith DeWaal, 2008). During this same time period there has been recognized increase in demand for

minimally processed fruits and vegetables (Rastogi *et al.* 2007; Sun-Waterhouse *et al.* 2014).

Concentrated fruit and vegetable production in Salinas Valley, California and Central Florida is necessary to meet consumer needs, but may explain the increase in foodborne illnesses caused by produce (Warriner *et al.* 2009). This shift away from small, locally sourced operations to large, capital-driven entities may have contributed to the spread of contaminated product across the nation, increasing the likelihood of multistate outbreaks due to national and international markets (Warriner *et al.* 2009). Pathogen detection and the importation of produce are recent changes to the produce industry, which may also contribute to more produce outbreaks (Tauxe *et al.* 1997; Brandl *et al.* 2006).

In 2007, California, the epicenter of the outbreak, and Arizona began the Leafy Greens Marketing Agreement (LGMA). This binding document placed stringent standards on growing practices such as microbial water quality and animal intrusion buffer zones. The number of field-based risk assessments intensified in the decade leading up to California's LGMA, a trend that would continue with the enactment of the Food Safety Modernization Act (FSMA) into law on January 4, 2011. With the signing of FSMA, the FDA led the most dramatic overhaul to food law since the 1916 Food Drug & Cosmetics Act. Combined laboratory- and field-based research focused on produce food safety has generated a wealth of information surrounding foodborne microorganisms in production areas. Recent studies along with framework documents established useful science-based standards (Harris *et al.* 2012). The final rule went into effect in 2015 with compliance dates going into effect over the next several years.

This chapter outlines basic pre-harvest food safety challenges, recent U.S. multistate outbreaks, and routes of contamination linked to fresh produce. There are numerous field trials in the literature, creating a strong foundation for effective environmental food safety research. In the sections to follow, a review of production practices (such as soil amendments, irrigation, and harvesting), which may increase the potential for foodborne outbreaks is discussed including bacterial pathogen survival and transmission to water, soil and plant milieu. Understanding factors involved in cross-contamination of produce in production areas will provide context, to generate a list of critical preventative measures to reduce the burden of foodborne illness associated with fruits and vegetables.

This review will mainly focus on two major foodborne pathogens because many of the recent outbreaks associated with *Salmonella* spp. and pathogenic *E. coli*. In 2010, U.S. foodborne illnesses linked to pathogenic *E. coli* and *Salmonella* infections cost approximately \$3.1 billion (Roos, 2010). Reducing enteric pathogens directly associated with pre-harvest management practices may limit the potential economic and health burden of a produce outbreak. Identifying effective mitigation strategies is the next step in pre-harvest microbial food safety research. This is a call to action for a collaborative effort, which evaluates current measures used to eliminate transmission of enteric pathogens through the consumption of raw agricultural commodities.

1.2 Produce Outbreaks (2012-2016)

To avoid redundancy in the literature, this section will discuss U.S. produce outbreaks occurring within a recent time frame, 2012-2016. A list of produce-linked outbreaks prior to 2012 has been compiled in recent publications (Warriner *et al.*

2009; CDC, 2017; Yeni *et al.* 2016). Due to short harvest and shelf-life periods for fruits and vegetables, the challenge for epidemiological investigations is pinpointing the contamination source linked to outbreaks (Berger *et al.* 2010). A specific source, in terms of how food came to be contaminated, may not always be determined.

From 2012 to 2014, there were 70 outbreaks involving produce commodities and the etiological agents *Salmonella* (40) and *E. coli* (30) that resulted in 2279 illnesses, 553 hospitalizations, and 7 deaths. Thirty of these outbreaks involved victims in multiple states and twenty-nine trace back investigations successfully identified the contaminated food. In seven of the twenty-nine outbreaks the contaminated food item was found in multiple states. In terms of geographical location for the non-multistate outbreaks, states that experienced the most outbreaks were Minnesota (7), Michigan (5), California (4), Pennsylvania (4), Connecticut, (2), Florida (2), Massachusetts (2), Illinois (2), and New York (2). Of the twenty-nine epidemiological investigations from 2012-2014, where outbreaks were associated with a single contaminated food source leafy greens (14), fruit (5), tomato (4), cucumbers (3), and sprouts (3) were most often identified. Produce outbreaks occur across the entire calendar year (2012-2014) but the months with the highest frequency were July (14), June (9), October (9), April (8), May (8). September and November had 5 outbreaks but only 3 occurred in the months of March and August, while two outbreaks per month during the winter months of December, January, and February were reported. (Information generated by using CDC Food tool available at <https://wwwn.cdc.gov/foodborneoutbreaks/>)

1.3 Routes of Pre-Harvest Contamination

Another distinctive feature of the 2006 *E. coli* outbreak was the fact that the epidemiologist matched the suspected strain from clinical samples to isolates found in packaged product, irrigation water, and feces (Sharapov *et al.* 2016). Outbreaks occur sometimes after an accumulation of chance events, often regarded as a “Perfect Storm” but the “smoking gun” was never determined. Microbial contamination can originate with contact to plant foliar tissues from agricultural water, soil, soil amendments, animals, and other fomites (Beuchat *et al.* 2002). Pre-harvest risks for contaminated produce include application of raw manure as fertilizer as well as the presence of domestic and wild animals in the field. However, each farm has a unique landscape, which highlights the problem associated with fresh produce contamination and risk assessment. Wild birds are easily able to migrate from compost piles to irrigation ponds and production areas. Many of the microbial risk factors associated with growing produce have been identified but due to the interaction between modes of transmission and environmental parameters problems persist. Similarly, these difficulties are encountered when performing basic field trials.

1.4 Agricultural Water

Water instability is at an all-time high in California, where nearly 6 million acres are dedicated to irrigated food crops (NASS, 2008). With water sources considered to be unacceptable due to climatic, contamination, financial, and geographical reasons, nation-wide fruit and vegetable producers have limited options when selecting their water supply. In 2008, the Census of Agriculture found 7 of the 10 million acres of U.S. commercial fruit, nut, and vegetables farms use irrigation methods to some degree (NASS, 2008).

Agricultural water is used for many purposes beyond irrigation such as fertilizer and pesticide application, frost protectant, dust control, and harvest aid. Fruit and vegetable production practices involving water have long been identified as a primary vector for food-surface contamination (Ruiz *et al.* 1987; Hillborn *et al.* 1999; Wheeler *et al.* 2005; Brandl *et al.* 2006; Harris *et al.* 2012). The microbial water quality is highly variable due to a variety of factors such as climatic events, season, adjacent land uses and wildlife, and water source (Heaton and Jones, 2008; Lazarova and Savoys, 2004; Ottoson and Stenstrom, 2003). Reviews of *E. coli* O157:H7 and *Salmonella* spp. in agricultural and surface water have demonstrated their presence in different water sources (Winfield and Groisman, 2003; Gerba and Smith 2005; Avery *et al.*, 2008). Water of poor microbiological quality used on raw agricultural commodities can facilitate raised levels of bacteria and potential of pathogen risk to consumers (Franz and van Bruggen, 2008; Benjamin *et al.* 2013; Won *et al.* 2013; Park *et al.* 2012; Castro-Rosas *et al.* 2012; Standing *et al.* 2013; Erickson, 2012).

In addition to contaminated farm water being recognized as one contributing source to the 2006 *E. coli* O157:H7 outbreak (CSFSAN FDA, 2007), the CDC investigation noted microbial differences between surface and ground water (Gelting, 2007). The importance of agricultural water sources is clearly stated in the Produce Safety Rule in which the FDA has determined the number of water samples needed as a function of the water type. Surface waters, which require more extensive monitoring, are open to wildlife and the environment. These are rivers, streams, and canals that are sourced for agricultural water. Intrusion of wildlife and farm animals into surface water sources, may perpetuate spread of foodborne pathogens to surrounding production areas (Solomon *et al.* 2002; Gorski *et al.* 2011). In two unrelated *E. coli*

O157:H7 outbreaks in 2005 and 2006, lettuce irrigated with water contaminated by cattle feces sickened 135 and 71 people in Sweden and the U.S., respectively, (CalFERT, 2008; Soderstrom *et al.* 2008). An outbreak strain of *Salmonella enterica* serovar St. Paul caused illnesses across 43 states in 2008 and was isolated from farm water and peppers from a U.S. importer and Mexican farm (Mody *et al.* 2011).

It is plausible for ground or well water to be exposed to contaminated water through recapture openings or poorly designed caps; however, ground water poses less of a risk compared to surface water when protected and maintained. *Salmonella* has reportedly shown long-term persistence from water reservoirs collected in two of the top three tomato-producing regions, Florida and the Eastern Shore Maryland (Bell *et al.* 2015). Extreme weather can cause flooding of produce fields and sub-surface wells (Oron *et al.* 2001; Ibekwe *et al.* 2004; Brandl *et al.* 2006; Gerba and Smith, 2005). As was the case in 1999, when a waterborne outbreak of *E. coli* O157:H7 occurred in Washington County, New York. Drinks and ice were prepared from a well believed to be contaminate by manure runoff contamination resulting in more than 1000 illnesses and two fatalities (Charatan, 1999).

In the Produce Safety Rule, municipal water is of least concern of the agricultural water sources and does not require a farm to test their water from a provider. However, contaminated municipal water led to the worst public health disaster in Canadian history (Salvadori *et al.* 2009). In Walkerton, Ontario, seven people died and more than 2000 fell ill from drinking water contaminated with *E. coli* O157:H7 and *Campylobacter jejuni* (Salvadori *et al.* 2009).

1.5 Animal intrusion

In the high profile 2006 outbreak, fecal samples collected from cattle and feral pigs tested positive for the outbreak strain, identifying another contributing factor to this outbreak (Sharapov *et al.* 2016; CalFERT, 2007). The presence of domestic and wild animals within production areas may introduce enteric pathogens (Cieslack *et al.* 1993; Jay *et al.* 2008; Jay-Russell *et al.* 2014). Significant fluctuations have been noted year-to-year, when fecal contamination originating from different animals was identified (Meays *et al.* 2006). For example, one year the majority of *E. coli* contamination was due to avian, deer, and canine; while in the following year most *E. coli* contamination was attributed to avian, bovine, and rodent species (Meays *et al.* 2006). Deer, feral pig, fox, rabbit, raccoon, goose and other migrating birds are common wild animals that can be found in production areas, though wildlife is known to change depending on geographical location. Wildlife intrusion may be difficult to predict and control. Both the LGMA and the FDA Produce Safety Rule state that areas of wildlife intrusion should be excluded from harvest due to risk of contamination. Research has generally supported the 5-ft no-harvest zone currently used as a means of managing produce safety risks concerning domestic and wild animal fecal contamination (LGMA, 2013).

Traditionally, cattle are considered the primary host for shiga-toxigenic *E. coli*, before being released into the environment via fecal droppings (Ferens *et al.* 2011). In 2011, an outbreak of *E. coli* O157:H7 in Oregon was linked to locally grown strawberries, which were contaminated by deer (Ladlier *et al.* 2013). Investigators found 11 deer samples all positive for *E. coli* O157:H7 in 5 different fields (Ladlier *et al.* 2013). Similar contamination events may occur with *Salmonella*, which has been regularly recovered from poultry but has multiple reservoirs in the environment, including

mammals, reptiles, and insects (Henzler *et al.* 1992; Wahlstrom *et al.* 2003; Meerburg *et al.* 2006; Holt *et al.* 2007; Wacheck *et al.* 2010).

After enteric pathogens are released into the non-host environment survival can be affected by extrinsic factors such as temperatures and acid stress, damaging UV, and nutrient competition. During warmer growing season, the presence of pathogenic *E. coli* has been shown to be significantly higher (Scaife *et al.* 2006). There are also important intrinsic elements encoding for attachment and multiple stress resistance genes, which may extend survival when exposed to inhospitable environments in soil, water, and on plants.

1.6 Worker Health and Hygiene

Another important component of pre-harvest food safety is worker health and hygiene. In the U.S, there are roughly 1 million hired farmworkers, consisting of mostly young migrant workers, most lack formal education and health care. Farmworkers' hands may contribute to microbial contamination of produce (Ravaliya *et al.* 2014), a large portion of the issues stem for poor hand washing (Berger *et al.* 2010). The produce industry is dependent on farmworkers performing excellent hygiene and handling practices, since the major fruit and vegetable crops are either completely or partially harvested by hand.

Farmworkers should be made aware of the illnesses and symptoms caused by foodborne pathogens, in addition to a general understanding of how etiological agents may be introduced to production areas (Guidance to minimize microbial food safety hazards for fresh fruits and vegetables, 1998). Safe handler and hygiene programs educate farm workers with the help of visual aids and address why these preventative measures are essential. They are taught not to hide illnesses or report to work if

displaying symptoms such as nausea, vomiting, diarrhea, and abdominal pain. While produce outbreaks have been linked to incidents involving sick handlers and poor hand washing (Hjertqvist *et al.* 2006, Falkenhorst *et al.* 2005, Wheeler *et al.* 2005). A limited number of studies have been conducted to address bacterial transmission associated with farm worker health and hygiene (Ravaliya *et al.* 2014). Viral infections such as those caused by norovirus and hepatitis A are indicative of farmworker related problems (Berger *et al.* 2010).

Training programs have emerged as the most effective approach to ensure food safety culture is achieved on farms. Inspection of sanitation practices during annual audits provides the current way to evaluate the impact of farmworker health and hygiene. Farm compliance is assessed through document inspection and field observation. Common citations range from undocumented trainings, water sources, and trainings to unsanitary facilities, equipment, and conditions in production areas.

In 2011, mandatory LGMA workshops were organized to teach safe handling and hygiene to farm supervisors. The next year, the LGMA's advisory board released its annual review. Farm audit scores reflected the impact of these worker trainings. The LGMA annual report, covering 500 audits, showed the number of citations (minor infractions to major deviations) due to worker practices a decreased from 2009 to 2012. In 2009 and 2010, there were 204 citations for worker practices. The following growing season of 2010-11, the number of citations was cut in half to 102, and then declined further to 80 citations in 2011-2012 (LGMA, 2013). Likewise, total citations from field observation (485 to 377) and sanitation (127 to 90) decreased over the three years of farm audits.

Minimizing the risk of foodborne illness through proper hand washing and sanitation programs is important to safe production of raw agricultural commodities. Training programs and subsequent audits highlight a means to implement and evaluate on-farm worker health and hygiene. A trained farmworker provides an additional resource to prevent and identify potential contamination.

1.7 Soil Amendments

Soil amendments are important to the production of fresh produce and a way to reuse organic farm waste in nutrient management plans (Harris *et al.* 2013). Soil amendments in the form of raw and composted animal manure, liquid, and slurries are applied to production areas, providing essential nutrients (nitrogen and phosphorus) and bedding material (Harris *et al.* 2012). However, use of untreated manure is a risk for contamination of fruits and vegetables (Franz and van Bruggen, 2008) Meat, fish and poultry production provide the most common animal-sourced soil amendments used for land application (Harris *et al.* 2012). Various types of soil amendments including animal husbandry waste from rodents, equine, and zoo animals may be used (Harris *et al.* 2012). Microbial survival and nutrient content may vary depending on animal source (Cote and Quessy 2005; Harris *et al.* 2012). Manure storage and treatment should be distanced from produce production and packing areas. Physical barriers are placed to limit spread by wind, flooding and leaching. It is common practice to spread manure on agricultural fields under animal manure management systems in the U.S. despite known contamination risks (Harris *et al.* 2012, Ziemer *et al.* 2010). Enteric pathogen survival in untreated and composted soil amendments has

been reviewed previously (Franz and van Bruggen 2008, Pachepsky *et al.* 2017, van Elsas *et al.* 2011, Wei and Kniel, 2011).

Composting is a process of raising the temperature to 131°F of manure piles for 3 days in order to breakdown organic materials into usable substrates. (Warriner *et al.* 2009). The resulting temperature abuse and alkaline stress inactivates microorganisms present in fecal material. Two methods for composting are generally used. The first method is passive or aging, where microbial content is reduced over time. The second method, active composting, is a meticulously controlled approach to treat animal manure. If performed correctly, enteric pathogens are inactivated over a number of days. Treatment times for both methods is contingent on dry matter, microbial loads, pH, moisture content, carbon and nitrogen ratio, aeration, and weather (Ingram and Milner, 2007; Leifert *et al.* 2008, Mannion *et al.* 2007, Semenov *et al.* 2007, You *et al.* 2006). The total overall organic material, incorporation method, environmental and soil conditions by which manure is applied influences enteric pathogen persistence in soil, and also, may affect long-term bacterial survival once soil amendments are present (Fenlon *et al.* 2000; Gagliardi and Kams, 2002; Hutchison 2004; Lau and Ingham, 2001, Nicholson *et al.* 2005; Semenov *et al.* 2009).

1.8 Implications of the Produce Rule: Guidelines for Pre-Harvest Regulations

The FDA Produce Safety Rule institutes science-based standards for growing, harvest, packing and holding raw agricultural commodities for human consumption provide oversight to U.S. and foreign farms (FDA, 2015). For water quality, the Produce Safety Rule adopted the acceptable water metrics originally based on Environmental Protection Agency (EPA) recreational water standards for *E. coli* concentrations in recreational water. Since surface water is most at risk for

contamination, more testing is required. Water from municipal/public water sources does not require additional testing outside of what is already done by the municipality and proven by certificate. Similar to surface water, untreated ground water requires testing to help evaluate risk. Water testing is used to calculate composite Geometric Mean and Statistical threshold data. In time, the FDA will develop an online calculator resource where water sample values can be generated from agricultural water testing.

Two categories of guidelines, no detectable *E. coli* or Geometric Mean/Statistical Threshold (<126/410 CFU generic *E. coli* per 100ml). The no detectable *E. coli* set for water used in involved in practices like handwashing or harvesting in which food contact surfaces are contacted must sample initially 4 times over a growing season. If acceptable, then one water sample will be taken annually. As for the numerical value generic *E. coli* criteria, the number of samples needed for will be demanding at first during initial survey comprised of 20 water samples. Then annually, 5 samples will only be needed because, Geometric Mean and Statistical Threshold are to be calculated using 5 new samples and 15 previous samples. The four most recent samples will be used to calculate the dataset used for the Statistical Threshold of 410. The GM is set at <126 CFU generic *E. coli* which measures the central tendency of water. Water samples collected will be averaged to indicate microbial levels relative to water metrics. Within individual samples, generic *E. coli* populations cannot exceed the STV of 410 CFU/100ml, by which the amount of variability of water due to rainfall or high river water is tabulated. All this information will go towards the development of a microbial water quality profile (Stoeckel *et al.* 2016). Compliance dates will vary based on farm size and according the Proposed

Rule released November 13th, 2017, all water related rules will allow an additional four year to comply (Produce Safety Alliance, 2017).

The Produce Safety Rule allows growers an opportunity to remediate water sources to lower generic *E. coli* levels to those stated in the final regulations. At this time, FDA does not mandate any prescribed method for treating water that is above the microbiological threshold. For water exceeding limits, recommended mitigation steps include water treatment, intervals between irrigation and harvest, or removal by washing must be in place within a year. However, stabilized compost and treated soil amendments now have allowable limits for *E. coli*, *Salmonella*, and *Listeria* along with prescribed two science-based methods for achieving compliance (FDA, 2015). FDA has currently has no objection to the National Organic Program (NOP standards) of at least 90 and 120 day interval between the application of raw manure as biological soil amendment for crops not in contact and in contact with soil under the FSMA final rule (FDA, 2015).

1.9 Weather and Food Safety

This section will address the potential for climate change to affect produce safety due to substantial increases in heavy precipitation events. As society begins to reduce its dependence on fossil, the produce industry is presented with new challenges, adhering to FSMA and global climate change. The inter-connected relationship between the incidence of foodborne disease and climate conditions has previously been recognized (Jacxsens *et al.* 2010, and Tirado *et al.*, 2010), suggesting that climate change is already impacting the occurrence of human pathogens within pre-harvest production areas.

Warm and moist conditions may enhance pathogen survival in production area, in addition to contaminated soil, water, and feces. As mentioned previously, issues that impact produce safety and quality is a multi-factorial. Heavy precipitation events

may contaminate edible portions of crops by splashing fecal material or dissemination by flooding and field run-off into irrigation water sources (Cevallos-Cevallos *et al.* 2012). Similarly, high-humidity conditions from dew, rain or irrigation water may enhance significant population growth for human pathogens (Brandl and Mandrell, 2002). While air temperature variation may be the most noticeable and measureable indicator of climate change, it is likely the number of heavy precipitation events are intensifying due to the interconnected nature of hydrological cycles (IPCC, 2014). According to IPCC assessment report, since 1950, the number of heavy precipitation events has increased in many regions more than it has decreased (IPCC, 2014). Further, based on averages from 1981-2000, the frequency of hurricanes in the North Atlantic has been above normal 9 out of 11 years with 2005 being a record-breaking season (IPCC. 2014). One possible explanation for the rise in heavy precipitation events could be the increase in overall atmospheric moisture content (Trenberth *et al.* 2007). The atmosphere contains more moisture as both air temperature and land precipitation increase (Trenberth *et al.* 2007). In turn raising soil moisture content as well (Warriner *et al.* 2009).

In field trials conducted at the University of Delaware in 2012-2013, survival of generic *E. coli* on tomatoes was impacted by rainfall and humidity more than by initial inoculum concentrations (ranged from 100 *E. coli* CFU/ml to 10,000 *E. coli* CFU/ml), applied to fruits. Warm and moist conditions may enhance pathogen survival in production areas harvesting following rainfall events may be delayed to ensure ample time for bacterial die-off. Awareness of bacterial contamination as a result of climatic events, specifically rainfall should be included in a Food Safety Plan and growers should be aware of the potential for contamination.

REFERENCES

1. Arnade, C., L. Calvin and F. Kuchler. 2010. Consumers' Response to the 2006 Foodborne Illness Outbreak Linked to Spinach.
<https://www.ers.usda.gov/amber-waves/2010/march/consumers-response-to-the-2006-foodborne-illness-outbreak-linked-to-spinach/>
2. Avery, L.M., A.P. Williams, K. Killham and D.L. Jones. 2008. Survival of *Escherichia coli* O157:H7 in waters from lakes, rivers, puddles and animal-drinking troughs. *Sci Total Environ.* 389(2-3):378-85.
3. Bell, R.L., J. Zheng, E. Burrows, S. Allard, C.Y. Wang, C.E. Keys, D.C. Melka, E. Strain, Y. Luo, M.W. Allard, S. Rideout, and E.W. Brown. 2015. Ecological prevalence, genetic diversity, and epidemiological aspects of *Salmonella* isolated from tomato agricultural regions of the Virginia Eastern Shore. *Front Microbiol.* 6:415
4. Benjamin, L., E.R. Atwill, M. Jay-Russell, M. Cooley, D. Carychao, L. Gorski, R.E. Mandrell. 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. *Int J Food Microbiol.* 165(1):65-76.
5. Berger, C.N., S.V. Sodha, R.K. Shaw, P.M Griffin, D. Pink, P. Hand, G. Frankel. 2010. *Environ Microbiol.* 12(9): 2385-2397.
6. Beuchat, L.R. 2002 Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect.* 4(4):413-23.
Review.

7. Brandl M.T. 2006; Fitness of human enteric pathogens on plants and implications for food safety. *Annu Rev Phytopathol.*44:367-92. Review.
8. Brandl, M.T. and R.E. Mandrell. 2002. Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl Environ Microbiol.* 68(7):3614-21.
9. CalFERT (California Food Emergency Response Team). 2008. Investigation of the Taco John's *Escherichia coli* O157:H7 outbreak associated with iceberg lettuce
10. Castro-Rosas J., J.F. Cerna-Cortés, E. Méndez-Reyes, D. Lopez-Hernandez, C.A. Gómez-Aldapa, T. Estrada-Garcia. 2012. Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *Int J Food Microbiol.* 156(2):176-80
11. Centers for Disease Control and Prevention. 2017. Multi-State Outbreaks Lists. <https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html>.
12. Charatan, F. 1999. *BMJ.* 1999. New York outbreak of *E coli* poisoning affects 1000 and kills two. 319(7214)
13. Cieslack, P.R., T.J. Barrett, P.M.Griffin, K.F. Gensheimer, G. Beckett, J. Buffington and M.G. Smith.1993. *Escherichia coli* O157:H7 infection from a manured garden. *Lancet.* 342(8867):367

14. Côté C. and S. Quessy. 2005. Persistence of Escherichia coli and Salmonella in surface soil following application of liquid hog manure for production of pickling cucumbers. J Food Prot. 68(5):900-905.
15. CSPI, Center for Science in the Public Interest. 2008. Outbreak Alert! 2008. https://cspinet.org/sites/default/files/attachment/outbreak_alert_2008_report_final.pdf
16. Don Stoeckel, D., G. Wall and E. Bihn. 2016. FSMA Produce Safety Rule Water Requirements: Insights to Get You Organized! Available at: <https://producesafetyalliance.cornell.edu/resources/educational-materials/fsma-produce-safety-rule-water-requirements-insights-get-you-organized> accessed 11/8/2017.
17. Erickson, M.C. 2012. Internalization of fresh produce by foodborne pathogens. Annu Rev Food Sci Technol.;3:283-310. doi: 10.1146/annurev-food-022811-101211. Epub 2012 Jan 11. Review.
18. Falkenhorst, G., L. Krusell, M. Lisby, S.B. Madsen, B. Böttiger and K. Mølbak. 2005 Imported frozen raspberries cause a series of norovirus outbreaks in Denmark. Euro Surveill.10(9)
19. FDA Guidance to minimize microbial food safety hazards for fresh fruits and vegetables, 1998
20. Fenlon, D.R., I.D. Ogden, A. Vinten and I. Svoboda. 2000. The fate of Escherichia coli and E. coli O157 in cattle slurry after application to land. Symp Ser Soc Appl Microbiol.(29):149S-156S.

21. Ferens, W.A. and C.J. Hovde. 2011. *Escherichia coli* O157:H7: animal reservoir and sources of human infection. *Foodborne Pathog Dis.* 2011. 8(4):465-87
22. Franz, E., A.V. Semenov, A.H.C. van Bruggen. 2008. Modeling the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil and the effect of intervention strategies. *Journal of Applied Microbiology*, 105 pp. 1569–1584
23. Gagliardi, J.V. and J.S. Karns. 2002. Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. *Environ Microbiol.* 4(2):89-96.
24. Gelting, R.J. 2007. A public health perspective on onsite wastewater systems. *J Environ Health.* 69(7):62-3
25. Gerba C.P. and J.E. Smith Jr. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. *J Environ Qual.* 34(1):42-8.
26. Gorski, L., C.T Parker, A. Liang, M.B. Cooley, M.T. Jay-Russell, et al. (2011) Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Applied and Environmental Microbiology* 77: 2734–2748.
27. Harris L. J., J. Bender, E.A. Bihn, T. Blessington, M.D. Danyluk, P. Delaquis, L. Goodridge, A.M. Ibekwe, S. Ilic, K.E. Kniel, J.T. Lejeune, D.W. Schaffner, D. Stoeckel, and T.V. Suslow. 2012. A framework for developing research protocols for evaluation of microbial hazards and controls during production

- that pertain to the quality of agricultural water contacting fresh produce that may be consumed raw. *J. Food Prot.* 75:2251-2273.
28. Harris, L.J., E.D. Berry, T. Blessington, M. Erickson, M. Jay-Russell, X. Jiang, K. Killinger, F.C. Michel, P. Millner, K. Schneider, M. Sharma, T.V. Suslow, L. Wang, R.W. Worobo. 2013. A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the application of untreated soil amendments of animal origin on land used to grow produce that may be consumed raw. *J Food Prot.* 76(6):1062-84.
29. Heaton, J.C. and K. Jones. 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J Appl Microbiol.* 104(3):613-26. Epub 2007 Oct 9.
30. Henzler, D.J. and H.M. Opitz. 1992. The role of mice in the epizootiology of *Salmonella enteritidis* infection on chicken layer farms. *Avian Dis.* 36(3):625-31.
31. Hilborn, E.D., J.H. Mermin, P.A. Mshar, J.L. Hadler, A. Voetsch, C. Wojtkunski, M. Swartz, R. Mshar, M.A. Lambert-Fair, J.A. Farrar, M.K. Glynn and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med.* 159(15):1758-1764.
32. Hjertqvist M., A. Johansson, N. Svensson, P.E. Abom, C. Magnusson, M. Olsson, K.O. Hedlund and Y. Andersson. 2006 Four outbreaks of norovirus

- gastroenteritis after consuming raspberries, Sweden, June-August 2006. *Euro Surveill.* 11(9)
33. Holt, P.S., C.J. Geden, R.W. Moore and R.K. Gast. 2007. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. *Appl Environ Microbiol.* 73(19):6030-6035.
34. Hutchison, M.L., L.D. Walters, A. Moore, K.M. Crookes and S.M. Avery. 2004 Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. *Appl Environ Microbiol.* 70(9):5111-8.
35. Ibekwe, A.M., P.M. Watt, P.J. Shouse, and C.M. Grieve CM. 2004. Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. *Can J Microbiol.* 50(12):1007-14.
36. Ingram, D.T. and P.D. Millner. 2007. Factors affecting compost tea as a potential source of *Escherichia coli* and *Salmonella* on fresh produce. *J Food Prot.*70(4):828-34
37. IPCC. 2014. *Climate Change 2014. Synthesis Report.* IPCC, Geneva, Switzerland.
38. Jacxsens, L., P.A. Luning, J.G.A.J. van der Vorst, F. Devlieghere, R. Leemans, M. Uyttendaele. 2010. Simulation modeling and risk assessment as tools to identify the impact of climate change on microbiological food safety — the

- case study of fresh produce supply chain. Food Research International, 43:1925–1935.
39. Jay, M.T and G. W. Wiscomb. 2008. Food Safety Risks and Mitigation Strategies for Feral Swine (*Sus scrofa*) near Agriculture Fields, Proc. 23rd Vertebr. Pest Conf.(R. M. Timm and M. B. Madon, Eds.) Published at Univ. of Calif., Davis. 2008. Pp. 21-25.
40. Jay-Russell, M.T., A.F. Hake, Y. Bengson, A. Thiptara, and T. Nguyen. 2014. Prevalence and Characterization of *Escherichia coli* and *Salmonella* Strains Isolated from Stray Dog and Coyote Feces in a Major Leafy Greens Production Region at the United States-Mexico Border PLoS One. 9(11): e113433.
41. Klein, S., and C. Smith DeWaal. (2008). Dirty dining. Center for Science in the Public Interest. Retrieved from: <http://cspinet.org/new/pdf/ddreport.pdf>
42. Laidler, M.R., M. Tourdjman, G.L. Buser, T. Hostetler, K.K. Repp, R. Leman, M. Samadpour and W.E. Keene *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. 2013 Clin Infect Dis. 57(8):1129-34.
43. Lau M.M. and S.C. Ingham SC. 2001 Survival of faecal indicator bacteria in bovine manure incorporated into soil. Lett Appl Microbiol. 33(2):131-6.
44. Lazarova, V. and P. Savoys. 2004 Technical and sanitary aspects of wastewater disinfection by UV irradiation for landscape irrigation. Water Sci Technol 50(2):203-9.

45. Leifert C., K. Ball, N. Volakakis and J.M. Cooper. Control of enteric pathogens in ready-to-eat vegetable crops in organic and 'low input' production systems: a HACCP-based approach. 2008. *J Appl Microbiol.* 105(4):931-950.
46. Liu, Z., S. Calvin and D. Middleton. 2007 Descriptive epidemiology of verotoxin-producing *E. coli* reported in Ontario, 1996-2005. *Can Commun Dis Rep.* 2007. 33(7):69-76.
47. Mannion, C., P.B. Lynch, J. Egan and F.C. Leonard. 2007. Seasonal effects on the survival characteristics of *Salmonella* Typhimurium and *Salmonella* Derby in pig slurry during storage. *J Appl Microbiol.* 103(5):1386-1392.
48. Meays, C.L., K. Broersma, R. Nordin, A. Mazumder and M. Samadpour. 2006. Spatial and annual variability in concentrations and sources of *Escherichia coli* in multiple watersheds. *Environ Sci Technol.* 40(17):5289-5296.
49. Meerburg, B.G., W.F. Jacobs-Reitsma, J.A. Wagenaar and A. Kijlstra. 2006. Presence of *Salmonella* and *Campylobacter* spp. in wild small mammals on organic farms. *Appl Environ Microbiol.* 72(1):960-962.
50. Mody, R.K., S.A. Greene, L. Gaul, A. Sever, S. Pichette, I. Zambrana, T. Dang, A. Gass, R. Wood, K. Herman, L.B. Cantwell, G. Falkenhorst, K. Wannemuehler, R.M. Hoekstra, I. McCullum, A. Cone, L. Franklin, J. Austin, K. Delea, C.B. Behraves, S.V. Sodha, J.C. Yee, B. Emanuel, S.F. Al-Khaldi, V. Jefferson, I.T. Williams, P.M. Griffin, and D.L. Swerdlow. 2011. National outbreak of *Salmonella* serotype saintpaul infections: importance of Texas

- restaurant investigations in implicating jalapeño peppers. *PLoS One*. 23;6(2):e16579.
51. Nicholson, F.A., S.J. Groves and B.J. Chambers. 2005. Pathogen survival during livestock manure storage and following land application. *Bioresour Technol*. 96(2):135-143.
52. Oron, G., R. Armon, R. Mandelbaum, Y. Manor, C. Campos, L. Gillerman, M. Salgot, C. Gerba, I. Klein, and C. Enriquez. 2001. Secondary wastewater disposal for crop irrigation with minimal risks. *Water Sci Technol*. 43(10):139-46.
53. Ottosson, J. and T.A. Stenström. 2003. Fecal contamination of greywater and associated microbial risks. *Water Res*. 37(3):645-55.
54. Ottosson, J. and T.A. Stenström. 2003. Growth and reduction of microorganisms in sediments collected from a greywater treatment system. *Lett Appl Microbiol*. 36(3):168-172.
55. Pachepsky, Y., M. Stocker, M.O. Saldaña and D. Shelton. 2017. Enrichment of stream water with fecal indicator organisms during baseflow periods. *Environ Monit Assess*. 189(2):51.
56. Park, S., S. Navratil, A. Gregory, A. Bauer, I. Srinath, M. Jun, B. Szonyi, K. Nightingale, J. Anciso, R. Ivanek. 2013. Generic *Escherichia coli* contamination of spinach at the preharvest level: the role of farm management and environmental factors. *Appl Environ Microbiol* 79:4347–4358.

57. Produce Safety Alliance 2017 <https://producesafetyalliance.cornell.edu/food-safety-modernization-act/produce-safety-rule-compliance-dates-timeline> accessed 11/9/17)
58. R. Mandrell. 2010. Enteric Human Pathogens Associated with Fresh Produce: Sources, Transport, and Ecology. *Microbial Safety of Fresh Produce*, pp.1-41
59. Rastogi, N.K., K.S. Raghavarao, V.M. Balasubramaniam, K. Niranjana and D. Knorr. Opportunities and challenges in high pressure processing of foods *Crit Rev Food Sci Nutr*. 2007. 47(1):69-112.
60. Ravaliya, K., J. Gentry-Shields, S. Garcia, N. Heredia, A. Fabiszewski de Aceituno, F.E. Bartz, J.S. Leon, L.A. Jaykus. 2014. Use of Bacteroidales microbial source tracking to monitor fecal contamination in fresh produce production. *Appl Environ Microbiol*. 80(2):612-617
61. Roos, R. USDA estimates E coli, Salmonella costs at \$3.1 billion. 2010. <http://www.cidrap.umn.edu/news-perspective/2010/05/usda-estimates-e-coli-salmonella-costs-31-billion>
62. Ruiz N, J.E. Marion, R.D. Miles, and R.B. Barnett. 1987. Nutritive value of new cultivars of triticale and wheat for broiler chick diets. *Poult Sci*. 66(1):90-7.
63. Salvadori, M.I., J.M. Sontrop, A.X. Garg, L.M. Moist, R.S. Suri, W.F. Clark WF. 2009. Factors that led to the Walkerton tragedy. *Kidney Int Suppl*. (112):S33-4

64. Scaife H.R., D. Cowan, J. Finney, S.F. Kinghorn-Perry and B. Crook. 2006. Wild rabbits (*Oryctolagus cuniculus*) as potential carriers of verocytotoxin-producing *Escherichia coli*. *Vet Rec.* 159(6):175-8.
65. Scharff, R.L. 2012. Economic burden from health losses due to foodborne illness in the United States. *J Food Prot.* 2012. 75(1):123-31.
66. Semenov, A.V., A.H.C. van Bruggen, L. van Overbeek, A.J. Termorshuizen, A.M. Semenov. 2007. Influence of temperature fluctuations on *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in cow manure. *FEMS Microbiology Ecology*, 60 (2007), pp. 419–428
67. Semenov, A.V., L. van Overbeek and A.H van Bruggen. 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Appl Environ Microbiol.* 75(10):3206-15
68. Sharapov UM, Wendel AM, Davis JP, Keene WE, Farrar J, Sodha S, Hyytia-Trees E, Leeper M, Gerner-Smidt P, Griffin PM, Braden C; OUTBREAK INVESTIGATION TEAM. 2006. Multistate Outbreak of *Escherichia coli* O157:H7 Infections Associated with Consumption of Fresh Spinach: United States. *J Food Prot.* 79(12):2024-2030.
69. Söderström, A., P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. Blide Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G. Allestam, I Hedenström, L. Ledet Muller, and Y. Andersson. 2008. A large

- Escherichia coli O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog Dis.* 5(3):339-49
70. Solomon, E.B., C.J. Potenski, and K.R. Matthews. 2002. Effect of irrigation method on transmission to and persistence of Escherichia coli O157:H7 on lettuce. *J Food Prot.* 65(4):673-6
71. Standing, T.A., E. du Plessis E, S. Duvenage, L. Korsten. 2013. Internalisation potential of Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica subsp. enterica serovar Typhimurium and Staphylococcus aureus in lettuce seedlings and mature plants. *J Water Health.* 11(2):210-23. doi: 10.2166/wh.2013.164
72. Sun-Waterhouse, D., G.I.N. Waterhouse, L. You, J. Zhang, Y. Liu, L. Ma, J. Gao J and Y. Dong. 2016 Transforming insect biomass into consumer wellness foods: A review. *Food Res Int.* 89(Pt 1):129-151.
73. Tauxe, R.V., 1997. Emerging foodborne diseases: an evolving public health challenge. *Emerg Infect Dis.* 3(4):425-34. Review.
74. The California Leafy Green Marketing Agreement. California Leafy Green Marketing Agreement: Commodity specific food safety guidelines for the production and harvest of lettuce and leafy greens. 2013. Available at: <http://www.caleafygreens.ca.gov/sites/default/files/California%20LGMA%20metrics%2008%2026%2013%20%20Final.pdf>. Accessed 22 November 2013.

75. The United States Department of Agriculture National Agricultural Statistics Service. (NASS) 2008. Available at:
https://www.nass.usda.gov/Publications/Ag_Statistics/2008/2008.pdf
76. The United States Food and Drug Administration Center for Food Safety and Applied Nutrition. (CFSAN FDA), 2007
<https://www.fda.gov/downloads/ICECI/UCM090298.pdf>
77. Tirado, M.C., R. Clarke, L.A. Jaykus, A. McQuatters-Gollop, J.M. Frank. 2010. Climate change and food safety: a review. *Food Research International*, 43:1745–1765
78. Trenberth, K.E., 2007. Warmer oceans, stronger hurricanes. Evidence is mounting that global warming enhances a cyclone's damaging winds and flooding rains. *Sci Am.* 297(1):44-51.
79. U.S Food and Drug Administration. Standards for the growing, harvesting, packing, and holding of produce for human consumption. (2015) Available at:
<https://www.federalregister.gov/articles/2015/11/27/2015-28159/standards-for-the-growing-harvesting-packing-and-holding-of-produce-for-human-consumption>. Accessed 14 March 2016.
80. U.S. Food and Drug Administration
FoodTool <https://www.cdc.gov/foodborneoutbreaks/>
81. van Elsas, J.D., A.V. Semenov, R. Costa and J.T. Trevors. 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects.

- 5(2): 173–183. Gastric abnormalities in cholera: epidemiological and clinical considerations.
82. Wacheck, S., M. Fredriksson-Ahomaa, M. König, A. Stolle, and R Stephan. 2010. Wild boars as an important reservoir for foodborne pathogens. *Foodborne Pathog Dis.* 7(3):307-12.
83. Wahlström. H., E. Tysén, E. Olsson Engvall, B. Brändström, E. Eriksson, T. Mörner and I. Vågsholm. 2003. Survey of *Campylobacter* species, VTEC O157 and *Salmonella* species in Swedish wildlife. *Vet Rec.* 153(3):74-80.
84. Warriner K., A. Huber, A. Namvar, W. Fan, K. Dunfield, L.T. Steve. Chapter 4 recent advances in the microbial safety of fresh fruits and vegetables. *Advances in Food and Nutrition Research*, vol. 57 Academic Press (2009), pp. 155–208
85. Wei, J., Y. Jin, T. Sims, K.E. Knierl. 2011. Internalization of murine norovirus 1 by *Lactuca sativa* during irrigation. *Appl Environ Microbiol.* 77(7):2508-2512
86. Wheeler, C., T.M. Vogt, G.L. Armstrong, G. Vaughan, A. Weltman, O.V. Nainan, V. Dato, G. Xia, K. Waller, J. Amon, T.M. Lee, A. Highbaugh-Battle, C. Hembree, S. Evenson, M.A. Ruta, I.T. Williams, A.E. Fiore and B.P. Bell. 2005. An outbreak of hepatitis A associated with green onions. *N Engl J Med.* 353(9):890-7.

87. Winfield M.D. and E.A. Groisman. 2003 Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl Environ Microbiol.* 69(7):3687-94.
88. Won G., P.J Schlegel, J.M. Schrock, and J.T. LeJeune. 2013. Absence of direct association between coliforms and *Escherichia coli* in irrigation water and on produce. *J. Food Prot.* 76:959-966.
89. Yeni, F., S. Yavaş, H. Alpas and Y. Soyer. 2016. Most Common Foodborne Pathogens and Mycotoxins on Fresh Produce: A Review of Recent Outbreaks. *Crit Rev Food Sci Nutr.* 56(9):1532-44. Review.
90. You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl Environ Microbiol.* 72(9):5777-83.
91. Ziemer CJ, Bonner JM, Cole D, Vinjé J, Constantini V, Goyal S, Gramer M, Mackie R, Meng XJ, Myers G, Saif LJ. 2010. Fate and transport of zoonotic, bacterial, viral, and parasitic pathogens during swine manure treatment, storage, and land application. *J Anim Sci.* 88(13 Suppl):E84-94

Chapter 2

USE OF GENERIC *E. COLI* AS AN INDICATOR OF POOR MICROBIOLOGICAL WATER QUALITY IN IRRIGATION WATER AND ON FRESH FRUITS AND VEGETABLES PRIOR TO HARVEST.

2.1 Abstract

Levels of *Escherichia coli* have been determined to be a gauge for the microbiological quality of irrigation water. In a study performed over two years, *E. coli* populations on leafy greens and tomatoes increased and then fluctuated following a one-time irrigation event with manure-contaminated water that contained from 100 to 10,000 CFU *E. coli*/ml. Significant changes in *E. coli* levels were observed over a 10-day harvest period following natural rainfall events ranging from 35 mm to 98 mm per rainfall event. These increases in *E. coli* levels were influenced by the growth of bacteria in the high moisture environment rather than following rainfall. Our field trials evaluated effects of rainfall events on *E. coli* populations on leafy green and tomatoes being cultivated on commercial farms in Maryland and Delaware. Following rainfall events, *E. coli* populations generally increased on tomatoes and on leafy greens during production, especially with spring crop of leafy greens. Levels tended to return to baseline of no detectable *E. coli* present within two days. *E. coli* populations were recovered sporadically and at low levels. This project provides on-farm validation of observations that rainfall may drive an increase in fecal indicator

bacterial populations. These data call into question the water quality profile standards used in the Produce Safety Rule of the Food Safety Modernization Act.

2.2 Introduction

Water is one of the most precious and limited natural resources on the planet. The most important use of this limited resource is for agricultural purposes, specifically for the irrigation and growth of crops which feed the world's growing population (Markland *et al.*, 2017). In the United States there are 330 million acres of land that are used for the production of food and other agricultural products (EPA, 2005). In 2008, the Census of Agriculture found that 7 of the 10 million acres of U.S. commercial fruit, nut, and vegetables farms use irrigation methods to some degree (NASS 2008). Agricultural water can also be used in fertilizer and pesticide application, frost protectant, dust control, and harvest aid. Production practices involving water have long been identified as a primary vector for fruit and vegetable surface contamination (Ruiz *et al.* 1987; Hillborn *et al.*, 1999; Wheeler *et al.*, 2005; Brandl *et al.*, 2006; Harris *et al.*, 2012). Reviews of *Escherichia coli* O157:H7 and *Salmonella* in agricultural and surface water have demonstrated their presence in different water sources (Winfield and Groisman, 2003; Gerba and Smith 2005). Water of poor microbiological quality used on raw agricultural commodities can facilitate raised levels of bacteria and potential of pathogen risk to consumers (Franz and van Bruggen, 2008; Benjamin *et al.*, 2013, Won *et al.*, 2013, Castro-Rosas *et al.*, 2012, Standing *et al.*, 2013, Erickson, 2012).

Since 1996, at least 130 U.S. foodborne-related outbreaks were attributed to fresh produce, with the majority of outbreaks of gastroenteritis linked to sprouts, melon, leafy greens and tomatoes. (Center for Disease Control and Prevention, 2013).

In a systemic review of scientific literature published up to 12 May 2010, more than 50% of the outbreaks of human illness following extreme water-related weather events reported heavy rainfall and flooding as the most common combination of events leading to contamination and illness (Cann *et al.*, 2013). The risk for spinach contamination with generic *E. coli* may increase by rain splashing or high humidity that precedes or follows rain events (Beuchat, 2006). Meanwhile, produce contaminated by *E. coli* may be made more severe by weather conditions, which facilitate the movement of *E. coli* to produce or increase persistence (Park *et al.*, 2015). The images of damage sustained during the 2017 hurricane in Mainland United States and the Caribbean is evidence of the power of strong storms. Extreme weather events can impact crop production and cause potential for foodborne illness due to fecal splashing and spreading (Kniel and Spanninger, 2017). Yet, climate change remains a polarizing issue affecting global policy and involving many experts across various disciplines analyzing its affects. (Kniel and Spanninger, 2017). Understanding the impacts of severe weather and how it may affect pre-harvest food safety is imperative for a safe and strong fresh produce industry.

Irrigation water was recognized as a contributing source of the 2006 spinach *E. coli* O157:H7 outbreak (CFSAN, 2007) where the CDC investigation noted microbial differences between surface and ground water (Gelting, 2007). Surface waters, which require more extensive monitoring than ground water, are open to wildlife and the environment. Surface waters include rivers, streams, and canals that are sourced for agricultural water. Intrusion of wildlife and farm animals into surface water sources, may perpetuate spread of foodborne pathogens to surrounding production areas (Solomon *et al.*, 2002; Gorski *et al.*, 2011). For example, in two

unrelated *E. coli* O157:H7 outbreaks in 2005 and 2006, lettuce irrigated with water contaminated by cattle feces sickened 135 and 71 people in Sweden and the U.S., respectively, (CalFERT, 2008). In 2008, an outbreak strain of *Salmonella enterica* serovar St. Paul caused illnesses across 43 states and was later isolated from farm water and peppers from a Mexican farm (Mody *et al.*, 2011). In 2010, a multistate outbreak of *E. coli* O145 linked to Romaine lettuce was presumably caused by human waste which had leaked from nearby septic tank to an irrigation canal in Yuma, AZ (Jay-Russell *et al.*, 2014).

Ground or well water can also be exposed to contaminated water through recapture openings or poorly designed caps; however, ground water poses less of a risk compared to surface water when protected and maintained. Extreme weather, such as excessive rainfall, can cause flooding of produce fields and sub-surface wells (Oron *et al.*, 2001, Ibekwe *et al.*, 2004, 2006; Brandl *et al.*, 2006; Gerba, 2009). As was the case in 1999, when a waterborne outbreak of *E. coli* O157:H7 occurred in Washington County, New York. Drinks and ice were prepared from a well following runoff contamination resulting in more than 900 illnesses and two fatalities (NY Dept. of Health, 2000).

E. coli is gram-negative bacterium that is a member of the *Enterobacteriaceae* family and resides in the gastrointestinal tracts of humans and animals (Winfield and Groisman, 2003). Both pathogenic and commensal strains exist within the microflora that reside in the mammalian gastrointestinal tract. It is important to understand the major similarities that are shared by both commensal *E. coli* and pathogenic *E. coli* subtypes. *E. coli* harbor genetically encoded proteins that allow them to be resistant to acidic conditions, which is necessary for survival inside the mammalian host (Blatner

et al., 1997, Lawrence and Ochman, 1998). Ishii *et al.* (2006) found *E. coli* is environmentally stable in temperate soil and water. Benjamin *et al.* (2013), sampled both water and sediment in both irrigation and non-irrigations water sources nearby. This study shows that water sample testing for fecal indicators may not be representative of actual *E. coli* populations and that slight disturbances caused by extreme rainfall or wind may dislodge deposited bacteria helping it to find a way into irrigation water. However, Erickson *et al.* (2010), reported that pathogenic *E. coli* was detected on 80-95% from spinach inoculated with 6 log CFU/ml *E. coli* O157:H7, 10-30% detection with 4 log CFU/ml inoculation, and 0% detected with 2 log CFU/ml *E. coli* O157:H7. These data highlights the variability that is seen in terms of persistence of *E. coli* in the environment. This project hopes to further understand water quality standards found within the Produce Safety Rule, while demonstrating the public health risk associated with using water contaminated with enteric pathogens on minimally processed fruit and vegetables.

2.3 Materials and Methods

2.3.1 Preparation of Manure Inoculum.

To determine the relationship between microbial contamination in water and microbial contamination on produce we used manure as our inoculum to confirm the assumption that there is a relationship between the extent of fecal material in irrigation water and the probability that produce will become contaminated. Fresh bovine manure samples were collected randomly (500g) from the University of Delaware

dairy. From this composite sample, a single 111 g sample was added to 11 L of deionized water establishing a 4 log CFU *E. coli*/ml concentration. The sample was agitated by hand for 2 minutes, allowed to sit for approximately ten minutes, agitated again for 2 minutes and serially diluted. *E. coli* was enumerated on TBX agar (Oxoid, UK) incubated at 37°C for 24 hrs.

2.3.2 Plant Preparation.

Seeds of Spinach (cv. Melody), Romaine Lettuce (cv. Parris Island), and Tomato (cv. BHN-602) were purchased from (Johnny's Selected Seeds, Winslow, ME). Seed trays were placed in the UDBG misting room (22-24°C, 85% humidity) until germination. Upon germination, plants were moved out to the UDBG main greenhouse (28-30°C, 60% humidity). Tomatoes were transplanted into 4" square traditional thin wall pots (Dillen Products, Middlefield, OH, USA) at 3 weeks. Plants were fertilized using Peter's Fertilizer (21-5-20) at a concentration of 200ppm nitrogen. A Dosatron (Dosatron International, Clearwater, FL, USA) set at a rate of 1:128 was used to apply fertilizer once per week while in the green house. At 6 weeks, seedlings were moved outside where they were hardened for 1 week. Plants were irrigated using drip irrigation tubing until full maturity. General recommendations practices for Delaware vegetable production were used for this research. Irrigation was performed using Drip Tape (Rain-Flo, East Earl, PA, USA) until full maturity. Tomatoes were grown in single rows with stakes (Honduran Pine Stakes) every 4' and plants evenly spaced at 18" apart. Tomatoes were grown in single bed; the rows were spaced 0.6 m apart and plants were evenly spaced at 0.3 m apart. Leafy greens were

grown in two rows of each bed, the rows were spaced 0.25 m and plants were spaced 0.1 m apart.

2.3.3 Plant Inoculation.

Leafy green and tomato plants (180 total per commodity, 12 groups of 15 with 3 different inoculum levels in triplicate) were inoculated at 4 log, 3 log, or 2 log CFU *E. coli*/ml. Non-inoculated (control) plants were treated identically with uncontaminated water. Plants were inoculated (250 ml) by overhead irrigation to simulate a one-time contamination event, saturating leaves and fruits of tomatoes and leafy greens for 5s using a sterilized graduated cylinder. Plant and fruit samples were collected on days 0, 1, 3, 5, 7 and 10.

2.3.4 Sampling and *E. coli* Enumeration.

Fruit samples (4 tomatoes [500-600g] from 4 different plants from each plot) and leaf samples (2 leaves from 4 different plants per plot) were chosen by using a random number selection system with low limits set at 1 and high limit set at 15 in Excel (Microsoft, WA, USA) and were weighed upon collection and the sample was agitated for 2 minutes. After processing, 1ml of sample were serially diluted performed in Phosphate Buffer Saline and 100 µl was plated on TBX agar in duplicate and incubated at 37°C and 42.5°C for 24h.

2.3.5 Confirmation of *E. coli* Isolates

A single colony from the TBX agar was then plated on EMB agar (Oxoid, UK) and incubated at 37°C for 24h. After incubation plates were checked for visible green sheen.

2.3.6 Quanti-Tray Colilert Enumeration

After processing, 100 mL tomato rinsate was added to a sterile bottle containing of one pack of Colilert powder shake until dissolved. Pour mixture into a Quanti-Tray/2000 and seal in an IDEXX Quanti-Tray Sealer (Westbrook, ME). Place the sealed tray in a 35±0.5°C incubator for 24 hours. Read results according to the Result Interpretation table below. Count the number of positive wells and refer to the MPN table provided with the trays to obtain a Most Probable Number.

2.3.7 Weather Data

A Delaware Environmental Observing System (DEOS) monitoring platform located adjacent to the experimental plot recorded the following data every 5 minutes: rainfall, temperature, solar radiation, and wind velocity

2.3.8 Statistical Analysis

Results are reported as the means (when possible) as log CFU/tomato. A one-way ANOVA analysis was performed to compare means within the data set across all inoculum levels and a linear regression model was generated using JMP 9 software (SAS Institute Inc., Cary, NC). All p values less than 0.05 were considered significant ($\alpha = 0.05$).

2.4 Results

2.4.1 Tomato Field Trials.

To evaluate *E. coli* populations on pre-harvest tomatoes, two repeated field trials were conducted from 2012 to 2013. Tomato samples (n=72) were harvested for up to 10 days post-inoculation (dpi) following a single application of manure-contaminated water. *E. coli* populations were recovered intermittently and weather conditions (rainfall, temperature, solar radiation, and wind velocity) were recorded.

In 2012, across all levels of contaminated plots the percentage of samples harvested that contained *E. coli* was 20.8% (15/72). At 0.1 dpi which was sampled directly after the contamination event onto tomato plants, *E. coli* was found to be below the detection limit (0.1 log CFU *E. coli*/tomato) on all tomatoes (0/3) harvested from three uncontaminated plots, but present in six (6/9) samples irrigated with either 4 log, 3 log, or 2 log CFU *E. coli*/ml. *E. coli* populations recovered from these fruits ranged from 0.25 to 1.4 log CFU *E. coli*/tomato and the highest overall *E. coli* populations (1.4 log CFU *E. coli*/tomato) was associated with the highest inoculation level (harvested from 4 log CFU *E. coli*/ml). On 1 dpi, *E. coli* was enumerated from a single (1/12) sample at 0.6 log CFU *E. coli*/tomato. On 3 dpi, low levels of *E. coli* were found on two (2/12) samples, ranging from 0.17 to 0.54 log CFU *E. coli*/tomato. Following harvest on 3 dpi, all plots experienced rainfall events of 37.6 mm and 22.4 mm on 3 and 5 dpi, respectively. Tomatoes sampled on 5 dpi were harvested under very wet conditions, however, one (1/12) sample had levels of *E. coli* (0.16 log CFU *E. coli*/tomato) above the detection limit (0.1 log CFU/tomato). On 7 dpi, *E. coli* was

enumerated from a single (1/12) sample at 0.22 log CFU *E. coli*/tomato. At the last sampling time, 10 dpi, two (2/12) samples had *E. coli* populations ranging from 0.26 to 0.40 log CFU *E. coli*/tomato. Matching PFGE patterns confirmed *E. coli* colonies found in the control plot and isolated from manure used for contamination were identical (Data not shown).

In contrast, the 2013 trial started with a large rainfall event of 78mm on 0.1 dpi, which occurred over a 2 hour period prior to the application of the contaminated water. For the remainder of the trial minor rainfall was experienced but nothing to the magnitude of the 2012 trial. Initial rainfall prior to the contamination event may explain the differences in populations of *E. coli* on harvested fruit from 2012 to 2013. Overall, *E. coli* was enumerated from 25.1% (18/72) of fruit surfaces over the 10-day period, which was slightly higher than 2012, where 19.4% (14/72) of samples had *E. coli* above the detection limit (0.1 log CFU *E. coli*/tomato). In the 2013 trials, tomato samples harvested immediately following inoculation had significantly greater *E. coli* counts when compared to all samples from 2012 regardless of inoculation level ($p < 0.05$).

For the 2013 tomato trial, following the contamination event 3.4 log CFU *E. coli*/tomato was enumerated from the highest inoculation level (4 log CFU *E. coli*/ml) and seven (7/12) samples from all inoculation groups on 0.1 dpi were found to contain *E. coli*, similar to the same number of samples positive for *E. coli* on 0.1 dpi during the 2012 trial. This sampling day was significantly different from the baseline established on 0 dpi where all harvested tomatoes were below the detection limit

($p < 0.05$). On 1 dpi, *E. coli* was enumerated from two (2/12) samples and populations were found to be 2.8 and 3.7 log CFU *E. coli*/tomato from 2 log and 3 log CFU *E. coli*/ml inoculated tomatoes, respectively. On 3 dpi, *E. coli* was found on one tomato (2.6 log CFU *E. coli*/tomato) sample from the 4 log CFU *E. coli*/ml group but no other samples had *E. coli* present above the detection limit. Tomatoes harvested on 5 dpi resulted in one (1/12) sample having levels of *E. coli* (3.6 log CFU *E. coli*/tomato) above the detection limit. On 7 dpi, *E. coli* was enumerated from a two (2/12) samples at similar concentrations from the 2 log and 3 log CFU *E. coli*/ml inoculated tomatoes at from 2.5 and 2.6 log CFU *E. coli*/tomato, respectively. At the last sampling time, 10 dpi, *E. coli* was not detected on any of the 12 samples harvested.

2.4.2 Leafy Green Field Trials

The initial leafy green trial was conducted in June 2013 (Newark, DE) followed by a repeated study in June 2014. Immediately following lettuce inoculation (0.1 dpi), samples ranged from 1.6 to 3.1 log CFU *E. coli*/leaf, with seven (7/12) samples positive for *E. coli*, while three (3/3) uncontaminated control plots were found to be below the detection limit. On 1 dpi, two (2/12) samples harvested from the 4 log CFU *E. coli*/ml inoculated lettuce were found to have populations of *E. coli* with an range of 0.1 to 2.8 log CFU *E. coli* /leaf. Interestingly, *E. coli* was found on harvested lettuce from a control plot but not present in six (6/6) plots inoculated with 2 or 3 log CFU *E. coli*/ml. Another example of *E. coli* disseminating to adjacent areas. At 3 dpi, five (5/12) samples were found with similar *E. coli* levels ranging from 1.9 to 2.1 log

CFU *E. coli*/leaf. *E. coli* populations persisted through 5 and 7 dpi, where populations again ranged from 0.2 to 2.8 log CFU *E. coli*/leaf. However, at 10 dpi, all twelve (12/12) samples harvested were found to contain *E. coli* ranging from 0.2-1.7 log CFU *E. coli*/leaf. It is likely that this time point was influenced by climatic conditions. The sampling of 10 dpi was preceded by 98 mm rainfall experienced on 7 dpi. On 10 dpi, lettuce and spinach were harvested in flood-like conditions and experienced 35 mm of precipitation in the form of rain. Overall, *E. coli* was enumerated from 52.7% (38/72) of lettuce samples,

Spinach trials were performed in parallel during June 2013. On 0.1 dpi, samples ranged from 0.1 to 2.9 log CFU *E. coli*/leaf, with seven (7/12) samples positive for *E. coli*. Two (2/3) uncontaminated control plots were found to have *E. coli* on spinach leaves while all three spinach samples harvested from the 3 log CFU *E. coli*/ml plots were found to be below the detection limit. On 1 dpi, one (1/12) sample harvested from the 4 log CFU *E. coli*/ml inoculated spinach was found to have low concentrations of *E. coli* with results at the 0.1 log CFU *E. coli* /leaf detection limit. At 3 dpi, six (6/12) samples were found to have *E. coli* levels ranging from 0.2 to 0.8 log CFU *E. coli*/leaf. Spinach harvested on 5 dpi resulted in four (4/12) samples having levels of *E. coli* ranging from 0.8 to 1.5 log CFU *E. coli*/leaf. Spinach samples harvested at 7 dpi showed *E. coli* populations in six (6/12) with levels of *E. coli* ranging from 0.5 to 1.6 log CFU *E. coli*/leaf. As was mentioned above, weather conditions on and prior to 10 dpi fields experienced rainfall over a four-day period, which resulted in all spinach samples harvested containing *E. coli* on 10 dpi. In total

from the 2013 trial, *E. coli* was enumerated from 48.6% (35/72) of spinach samples. This was somewhat anticipated, due to weather conditions and the inability to control all variables that may influence microbiological trials in the pre-harvest environment.

In 2014, following lettuce inoculation (0.1 dpi), samples ranged from 1.4 to 4.7 log CFU *E. coli*/leaf, with nine (9/12) with detectable levels of *E. coli*, while three (3/3) uncontaminated control plots were found to be below the detection limit. On 1 dpi, seven (7/12) samples harvested, all from inoculated lettuce, were found to have populations of *E. coli* with an range of 1.4 to 3.7.8 log CFU *E. coli* /leaf. Again, all three (3/3) uncontaminated control plots were found to be below the detection limit. At 3 dpi, six (6/12) samples were found with similar *E. coli* levels ranging from 0.1 to 2.5 log CFU *E. coli*/leaf. Lettuce harvested on 5 dpi resulted in five (5/12) samples having levels of *E. coli* ranging from 1.3 to 2.5 log CFU *E. coli*/leaf. Lettuce samples harvested at 7 dpi showed *E. coli* populations in nine (9/12) with levels of *E. coli* ranging from 0.1 to 1.8 log CFU *E. coli*/leaf. At 10 dpi, eight (8/12) samples harvested were found to contain *E. coli* ranging from 0.2-3.5 log CFU *E. coli*/leaf.

For the 2014 spinach results, (20/72) 27.8% of spinach harvested was found to have *E. coli* much lower than the previous year of 48.6% and well below the 61.1% of lettuce samples containing *E. coli* during the 2014 trial. On 0.1 dpi, samples ranged from 3.4 to 4.8 log CFU *E. coli*/leaf, with seven (7/12) samples positive for *E. coli*. On 1 dpi, one (1/12) sample harvested from the 3 log CFU *E. coli*/ml inoculated spinach was found to have high concentrations of *E. coli* with results at the 4.5 log CFU *E. coli* /leaf detection limit. At 3 dpi, three (3/12) samples were found to have *E. coli* levels

ranging from 1.9 to 3.7 log CFU *E. coli*/leaf. Spinach harvested on 5 dpi resulted in four (3/12) samples having levels of *E. coli* ranging from 2.2 to 3.1 log CFU *E. coli*/leaf. Spinach samples harvested at 7 dpi showed *E. coli* populations in seven (7/12) with levels of *E. coli* ranging from 1.1 to 2.9 log CFU *E. coli*/leaf. At 10 dpi, nine (9/12) samples harvested were found to contain *E. coli* ranging from 2.6-4.9 log CFU *E. coli*/leaf. The sampling of 10 dpi was preceded by 61 mm rainfall experienced over a four-day period.

2.5 Discussion

Overall, our results found that *E. coli* can survive on plant surfaces and in water. Both the 2013 leafy green trials, the following year, 2014, lettuce results again showed higher percentage of samples with *E. coli* compared to spinach. In 2014, 61.7% (44/72) of harvested lettuce samples had populations of *E. coli*, while less than a third of (27.8% [20/72]) spinach samples were only found to *E. coli* above the detection limit. This may be explained by the differences in plant structure and surface area. Lettuce used in this trial has outer leaves and contained more overall leafy material, while spinach has individual leaves which are not in contact with each other and leaves did not contact the soil surface. Consistent to the results presented in the current study, Hutchinson *et al.* (2008) found greater numbers of pathogenic *Salmonella* and *E. coli* on lettuce compared to spinach when irrigated with water at 10^5 CFU/ml 1-2 hours after inoculation. A previous study by Ingham *et al.*, (2005) used field trials to test leafy greens and herbs in order to attempt to determine indicator

concentrations of *E. coli* for leafy greens, which were less than 1 to 1.5 log CFU/g *E. coli*. Since leafy greens are low-lying in proximity to the ground relative to tomatoes, extreme weather affecting regrowth or splashing of *E. coli* may not need to be as severe as a rainfall event impacting tomatoes. Meaning rainfall amounts from 20-50 mm may cause a growth of *E. coli* but not have any impact on tomatoes. Also, the level of *E. coli* present in the field may need to be significantly higher to facilitate growth.

Field trial results have revealed associations between generic *E. coli* concentrations and fluctuations in rainfall, wind, temperature, and solar radiation. With weather experienced during each trial, rainfall had the most obvious impact on the presence of generic *E. coli* on leafy greens. This may be due to dispersal of inoculum throughout the field caused by flooding, proximity to the ground and soil splash. Moreover, rainfall events may create a conducive environment for *E. coli*. These results show irrigation metrics may be more useful to control bacterial loads on leafy greens compared to tomatoes, in particular after large rainfalls similar to the storm that occurred during the leafy green trial. In addition, the relationship between the native microbiota and *E. coli* is another important variable that needs to be studied to further understand the fate of foodborne pathogens and indicator organisms outside the host. The data gathered in this project has been used to help generate science-based metric used in the water quality section of Produce Safety Rule, enhancing our knowledge of the part fruits and vegetables have in the fate of *E. coli* in non-host environments.

Table 1. *E. coli* positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2014.

<i>E.coli</i>	Table 1 <i>E. coli</i> positive samples collected from leaf tissue enumerated on TBX											
	Spinach Spring 2014						Lettuce Spring 2014					
	Days (6/4/14-6/14/14)											
	0.1	1	3	5	7	10	0.1	1	3	5	7	10
10 ⁰	-	-	+	2/3	-	2/3	+	+	+	-	1/3	1/3
10 ²	1/3	-	+	+	3/3	2/3	3/3	1/3	+	1/3	2/3	2/3
10 ³	3/3	1/3	1/3	1/3	2/3	2/3	3/3	3/3	3/3	1/3	3/3	2/3
10 ⁴	3/3	+	2/3	+	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3

+Positive by Enrichment on EMB
Spinach Spring 2014 samples (20/72) 27.8%
Lettuce Spring 2014 samples (44/72) 61.1%

Table 2. *E. coli* positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2013.

<i>E. coli</i>	Table 2 <i>E. coli</i> positive samples collected from leafy tissue enumerated on TBX											
	Spinach Spring 2013						Lettuce Spring 2013					
	Days (5/31/13-6/10/13)											
	0.1	1	3	5	7	10	0.1	1	3	5	7	10
10 ⁰	2/3*	-	1/3	1/3	2/3*	3/3*	-*	1/3*	-	-	1/3	3/3*
10 ²	2/3*	-	1/3*	-	3/3	3/3*	2/3*	-*	1/3*	1/3*	2/3*	3/3*
10 ³	-*	-	1/3*	2/3	-	3/3*	2/3*	-	1/3	-	2/3*	3/3*
10 ⁴	3/3*	1/3*	3/3*	1/3*	1/3*	3/3*	3/3*	2/3*	3/3*	2/3*	3/3*	3/3*

*Positive by Colilert MPN
Spinach Spring 2013 samples (35/72) 48.6 %
Lettuce Spring 2013 samples (38/72) 52.7%

Table 3 *E. coli* positive samples collected from fruit and leaf tissue enumerated on TBX in Delaware in 2012.

UD	Table 3 <i>E. coli</i> positive samples collected from fruit tissue enumerated on TBX											
	Tomato Summer 2012						Tomato Summer 2013					
	Days (8/31/12-9/10/12)						(8/12/13-8/23/13)					
<i>E. coli</i>	0.1	1	3	5	7	10	0.1	1	3	5	7	10
10 ⁰	-	1/3*	0*	-	1/3*	1/3*	-	-	-	-	-	-
10 ²	0*	0*	0*	1/3	0*	1/3	2/3	1/3*	-	1/3	2/3	-
10 ³	1/3*	1/3*	2/3*	-	0*	2/3*	2/3*	1/3*	-	-	2/3	-
10 ⁴	2/3*	-	1/3	0*	0*	0*	3/3*	-	1/3*	-	-	-

*Positive by Colilert MPN Tomato Summer 2012 samples (14/72)
 19.4% Tomato Summer 2013 samples (18/72) 25.0%

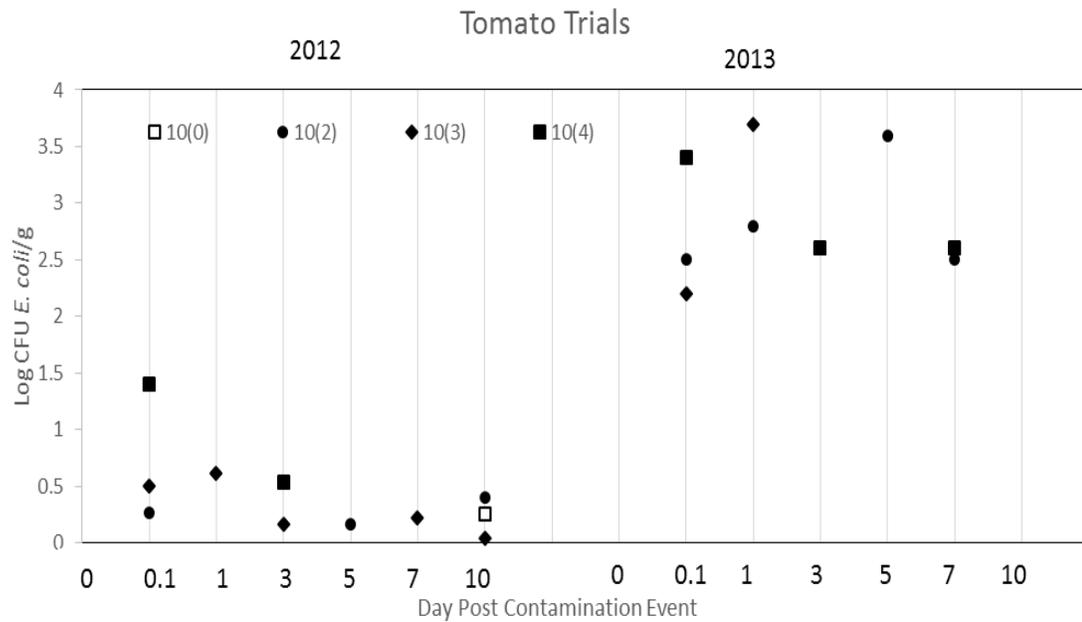


Figure 1. *E. coli* concentrations from tomatoes harvested and enumerated on TBX in Delaware from 2012 to 2013.

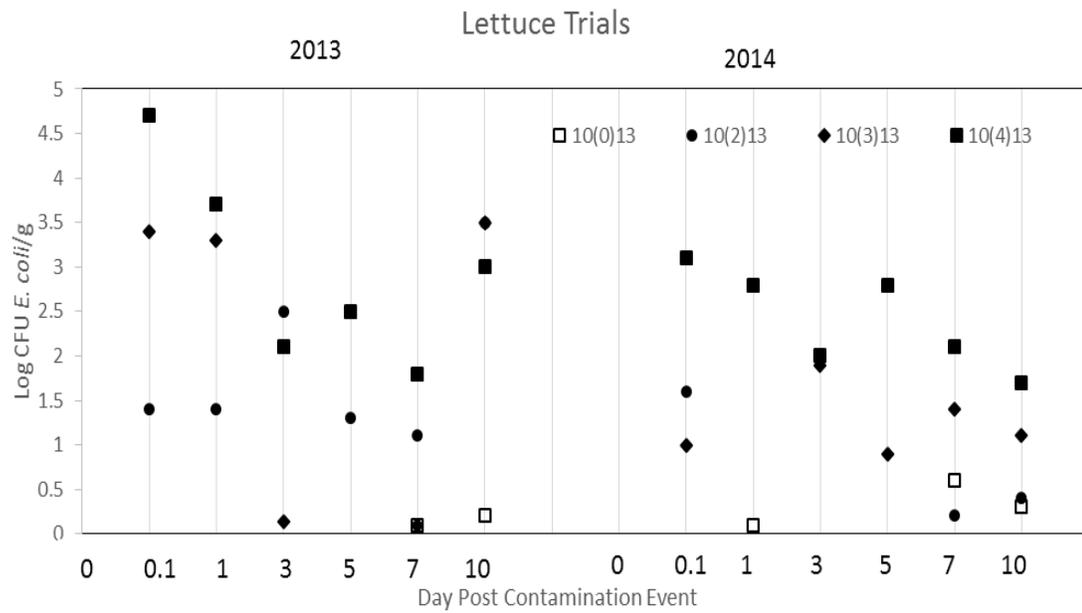


Figure 2. *E. coli* concentrations from lettuce plants harvested and enumerated on TBX in Delaware from 2013 to 2014.

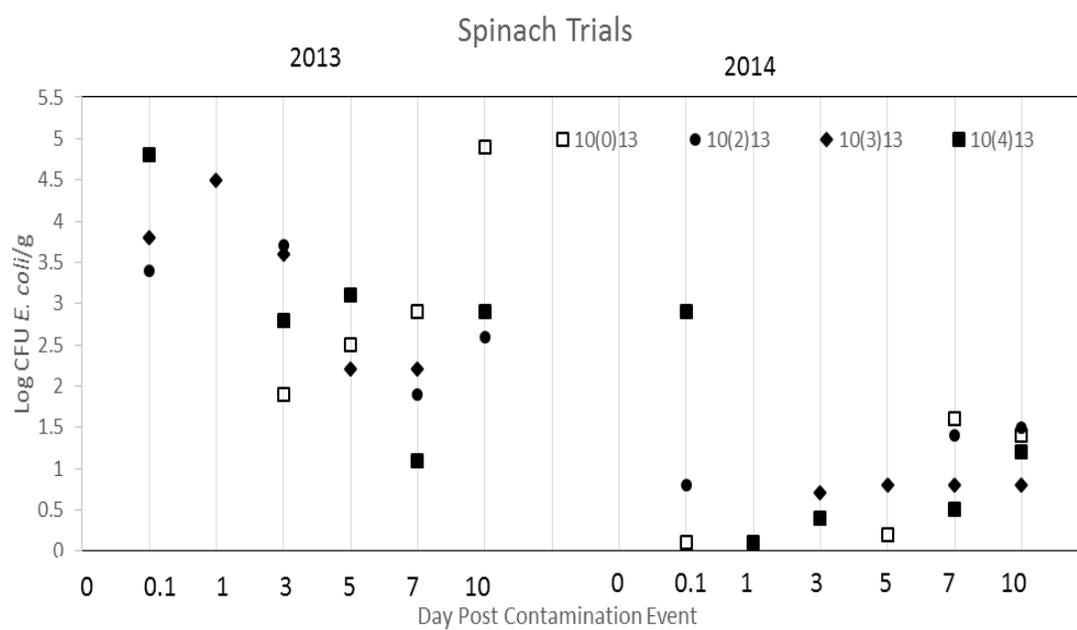


Figure 3. *E. coli* concentrations from spinach plants harvested and enumerated on TBX in Delaware from 2013 to 2014.

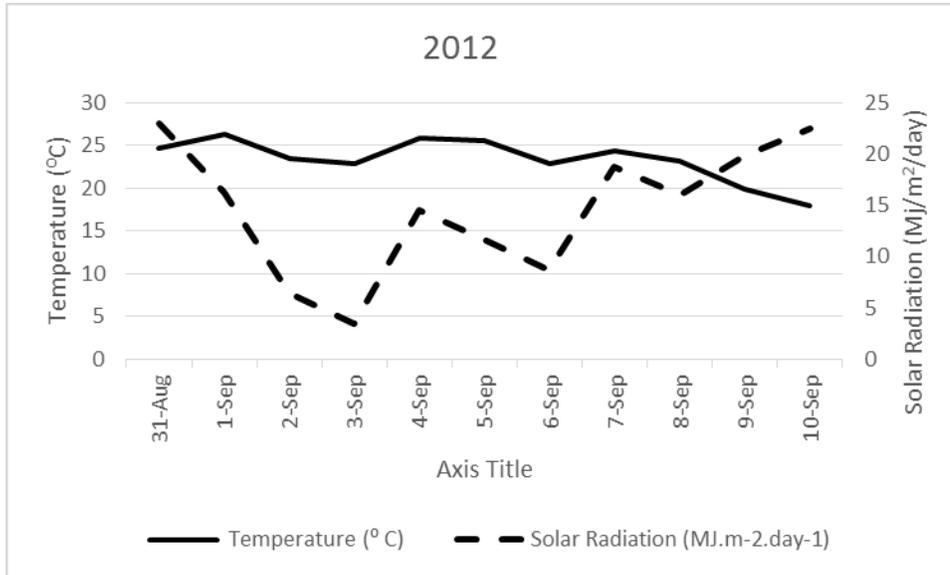


Figure 4. The average air temperature (solid line) and radiation (dashed line) from 31 August through 10 September 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with *E. coli*.

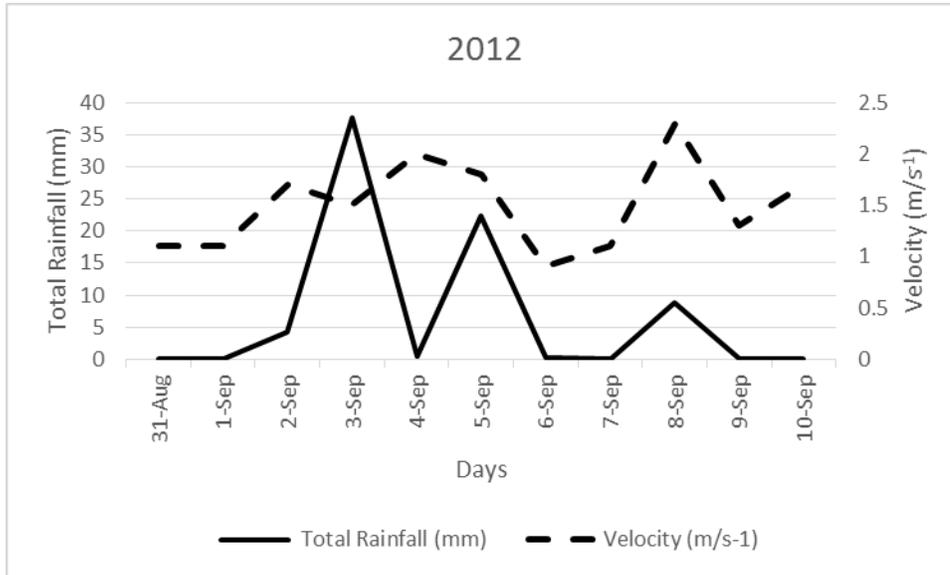


Figure 5. The total rainfall (solid line) and average wind velocity (dashed line) from 31 August through 10 September 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with *E. coli*.

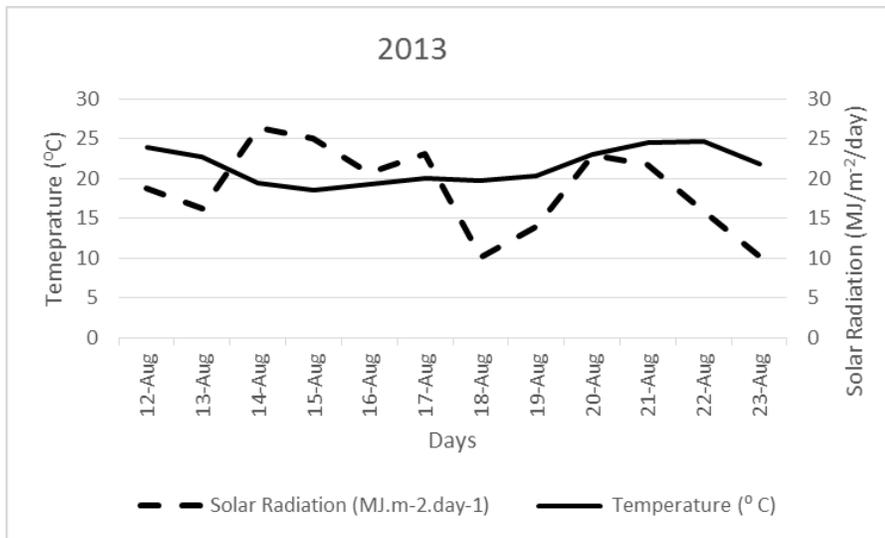


Figure 6. The average air temperature (solid line) and radiation (dashed line) from 12 August through 23 August 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with *E. coli*.

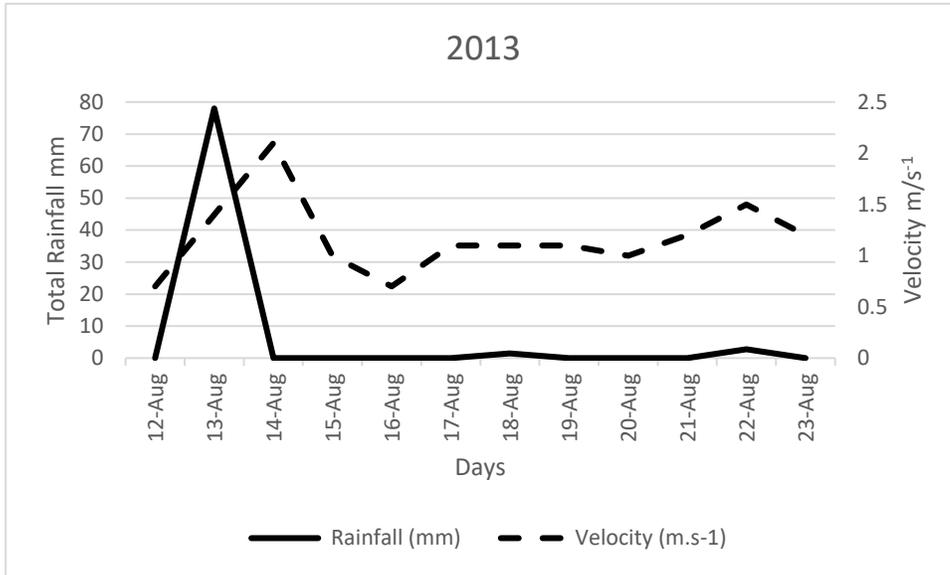


Figure 7. The total rainfall (solid line) and average wind velocity (dashed line) from 12 August through 23 August 2012 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with *E. coli*.

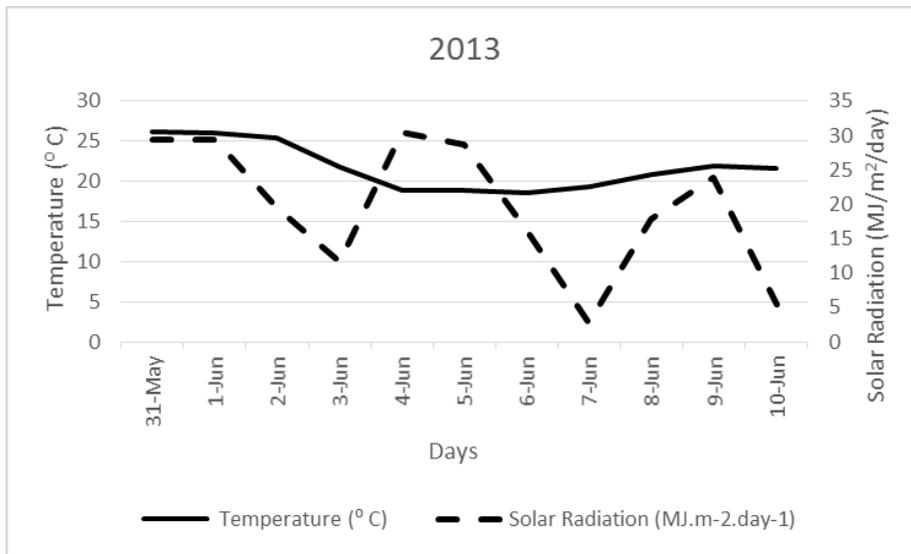


Figure 8. The average air temperature (solid line) and radiation (dashed line) from 31 May through 10 June 2013 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with *E. coli*.

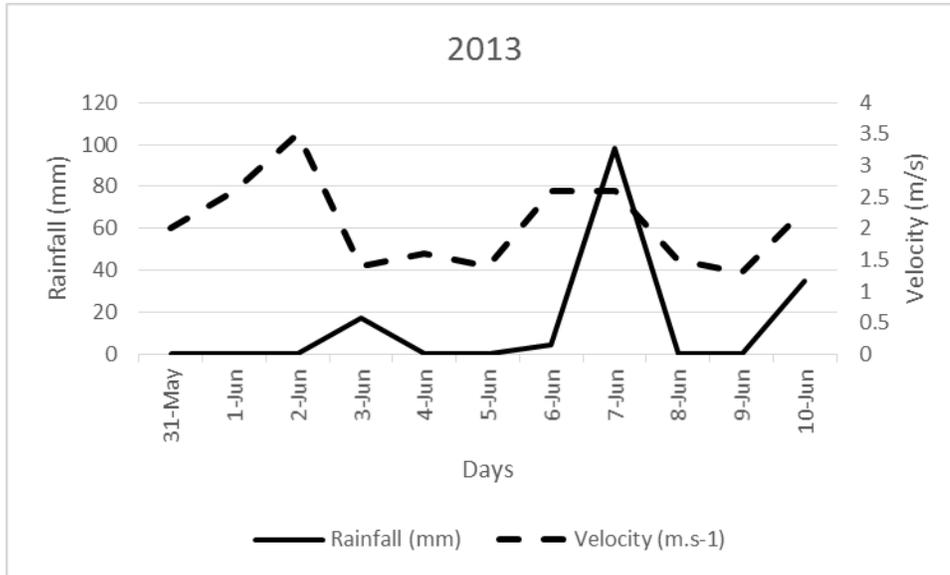


Figure 9. The total rainfall (solid line) and average wind velocity (dashed line) from 31 May through 10 June 2013 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with *E. coli*.

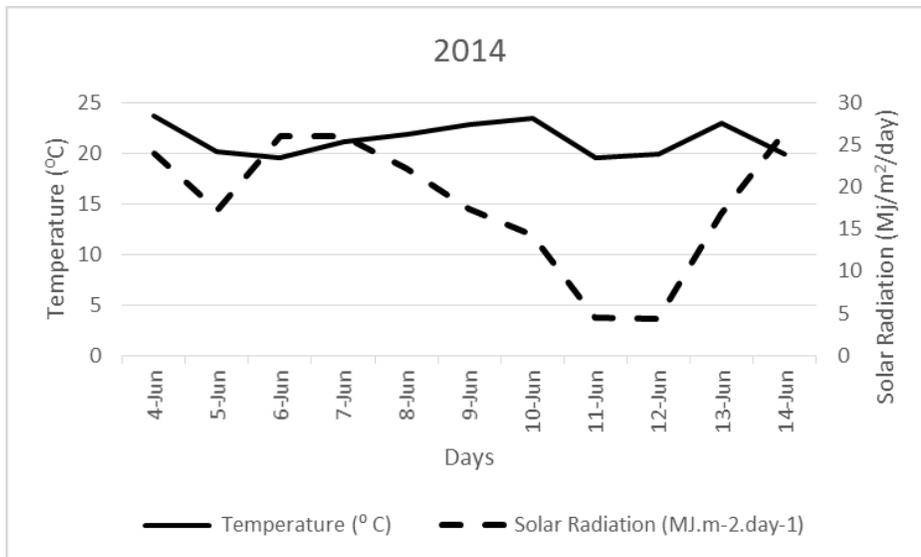


Figure 10. The average air temperature (solid line) and radiation (dashed line) from 4 June through 14 June 2014 at the University of Delaware Farm (Newark, DE, USA), where spinach and lettuce plants were cultivated and irrigated with water inoculated with *E. coli*.

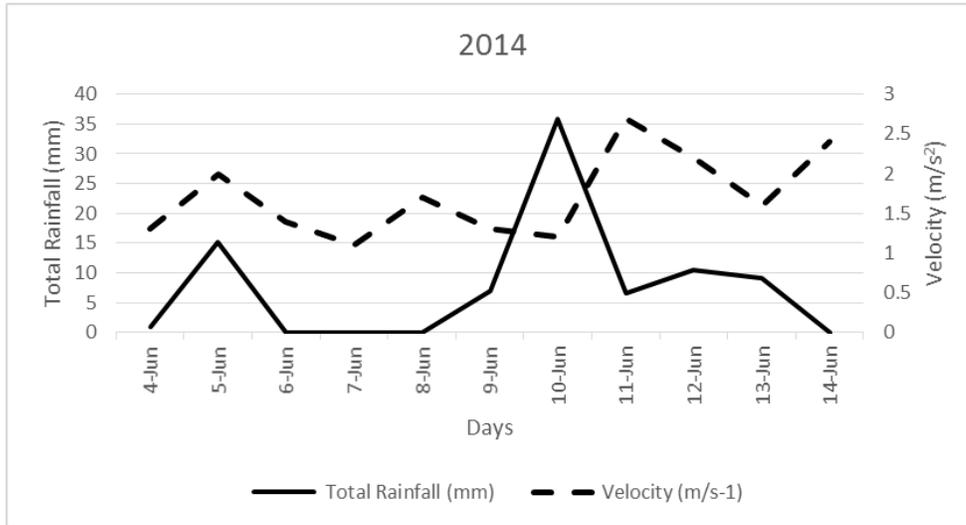


Figure 11. The total rainfall (solid line) and average wind velocity (dashed line) from 4 June through 14 June 2014 at the University of Delaware Farm (Newark, DE, USA), where tomato plants were cultivated and irrigated with water inoculated with *E. coli*.

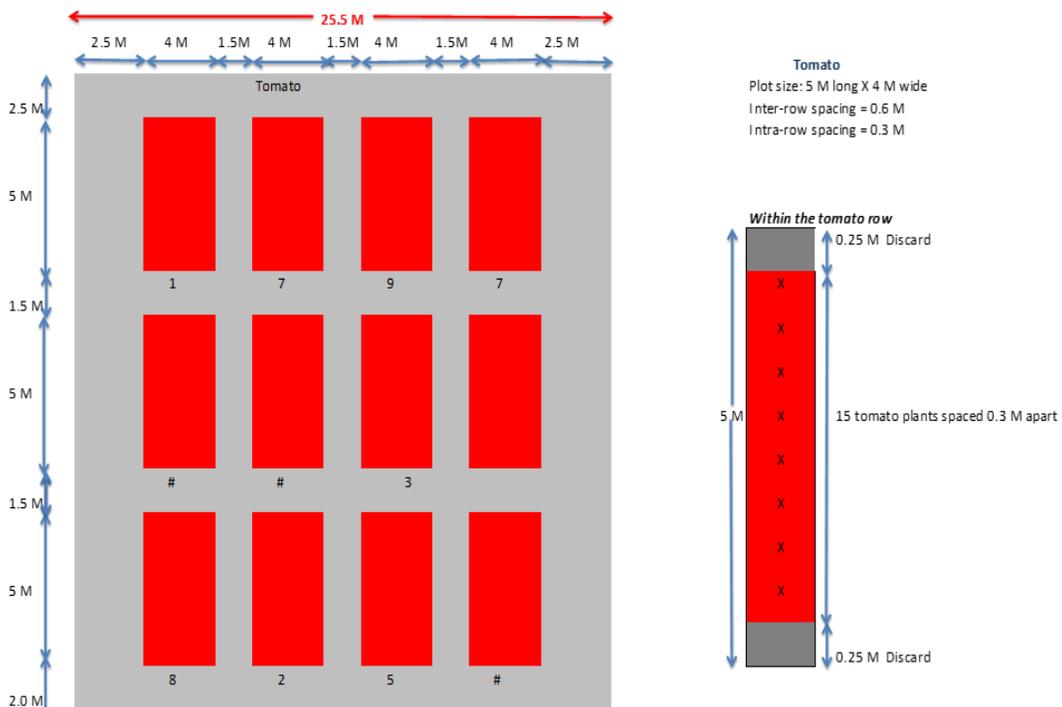


Figure 12. Experimental layout and dimensions for 12 plots containing tomato plants.

REFERENCES

1. Bell, R.L., J. Zheng, E. Burrows, S. Allard, C.Y. Wang, C.E. Keys, D.C. Melka, E. Strain, Y. Luo, M.W. Allard, S. Rideout, and E.W. Brown. 2015. Ecological prevalence, genetic diversity, and epidemiological aspects of *Salmonella* isolated from tomato agricultural regions of the Virginia Eastern Shore. *Front Microbiol.* 6:415
2. Benjamin, L., E.R. Atwill, M. Jay-Russell, M. Cooley, D. Carychao, L. Gorski, and R.E. Mandrell. 2013, Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. *Int J Food Microbiol.* 165(1):65-76.
3. Beuchat, L.R. 2002 Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect.* 4(4):413-23. Review.
4. Blattner, F.R., G. Plunkett 3rd, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, J.D. Glasner, C.K.Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau, and Y. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science.* 277(5331):1453-62.
5. Brandl M.T. 2006; Fitness of human enteric pathogens on plants and implications for food safety. *Annu Rev Phytopathol.*44:367-92. Review.
6. CalFERT (California Food Emergency Response Team). 2008. Investigation of the Taco John's *Escherichia coli* O157:H7 outbreak associated with iceberg lettuce

7. Cann K.F., D.R. Thomas, R.L. Salmon, A.P. Wyn-Jones, D. Kay. 2013 Extreme water-related weather events and waterborne disease. *Epidemiol Infect* 141:671-686.
8. Castro-Rosas, J., J.F. Cerna-Cortés, E. Méndez-Reyes, D. Lopez-Hernandez, C.A. Gómez-Aldapa, T. Estrada-Garcia. 2012. *Int J Food Microbiol.*156(2):176-80.
9. Caugant, D.A., B.R. Levin, and R.K. Selander. Genetic diversity and temporal variation in the *E. coli* population of a human host. *Genetics*. 1981.98(3):467-90.
10. Charatan, F. 1999. *BMJ*. 1999. New York outbreak of *E coli* poisoning affects 1000 and kills two. 319(7214)
11. EPA. 2005. Protecting water quality from agricultural runoff.
https://www.epa.gov/sites/production/files/2015-09/documents/ag_runoff_fact_sheet.pdf.
12. Erickson, M.C. 2012. Internalization of fresh produce by foodborne pathogens. *Annu Rev Food Sci Technol.*;3:283-310. doi: 10.1146/annurev-food-022811-101211. Epub 2012 Jan 11. Review.
13. Franz, E. and A.H. van Bruggen. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Crit Rev Microbiol*. 34(3-4):143-61.
14. Gelting, R.J. 2007. A public health perspective on onsite wastewater systems. *J Environ Health*. 69(7):62-3

15. Gerba C.P. and J.E. Smith Jr. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. *J Environ Qual.* 34(1):42-8.
16. Gorski, L., C.T. Parker, A. Liang, M.B Cooley, M.T Jay-Russell, A.G. Gordus, E.R. Atwill, and R.E. Mandrell. 2011. Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Appl Environ Microbiol.* 77(8):2734-48.
17. Harris, L.J., J. Bender, E.A. Bihn, T. Blessington, M.D. Danyluk, P. Delaquis, L. Goodridge, A.M. Ibekwe, S. Ilic, K. Kniel, J.T. Lejeune, D.W. Schaffner, D. Stoeckel and T.V. Suslow. 2012 A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the quality of agricultural water contacting fresh produce that may be consumed raw. *J Food Prot.* 75(12):2251-73.
18. Heaton, J.C. and K. Jones. 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J Appl Microbiol.* 104(3):613-26. Epub 2007 Oct 9.
19. Hilborn, ED, J.H Mermin, P.A. Mshar, J.L Hadler JL, A. Voetsch , C. Wojtkunski, M. Swartz, R. Mshar R, M.A. Lambert-Fair, J.A. Farrar, M.K Glynn, and L. Slutsker. 1999 A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med.* 1999 Aug 9-23;159(15):1758-64.

20. Ibekwe, A.M., P.M. Watt, P.J. Shouse, and C.M. Grieve CM. 2004. Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. *Can J Microbiol.* 50(12):1007-14.
21. Jay-Russell, M.T., A.F. Hake, Y. Bengson, A. Thiptara, and T. Nguyen. 2014. Prevalence and Characterization of *Escherichia coli* and *Salmonella* Strains Isolated from Stray Dog and Coyote Feces in a Major Leafy Greens Production Region at the United States-Mexico Border *PLoS One.* 9(11): e113433.
22. Kniel, KE. and P. Spaninger. 2017. Preharvest Food Safety under the influence of Climate Change. *Microbiol. Spectrum.*5(2):pFS-0015-2015.
23. Lawrence, J.G. and H. Ochman. 1998. Molecular archaeology of the *Escherichia coli* genome. *Proc Natl Acad Sci U S A.* 95(16):9413-7.
24. Lazarova, V. and P. Savoys. 2004. Technical and sanitary aspects of wastewater disinfection by UV irradiation for landscape irrigation. *Water Sci Technol.* 50(2):203-9.
25. Markland, S.M., D. Ingram, K.E. Kniel and M. Sharma. 2017. Water for Agriculture: the Convergence of Sustainability and Safety. *Microbiology Spectrum.* doi: 10.1128/microbiolspec.PFS-0014-2016.
26. Mody, R.K., S.A Greene, L. Gaul, A. Sever, S. Pichette, I. Zambrana, T. Dang, A. Gass , R. Wood, K. Herman, L.B. Cantwell, G. Falkenhorst, K. Wannemuehler, R.M. Hoekstra, I. McCullum, A. Cone, L. Franklin, J. Austin, K. Delea, C.B. Behraves, S.V. Sodha, J.C. Yee, B. Emanuel, S.F. Al-Khaldi, V. Jefferson, I.T. Williams, P.M. Griffin, and D.L. Swerdlow. 2011. National outbreak of

- Salmonella serotype saintpaul infections: importance of Texas restaurant investigations in implicating jalapeño peppers. *PLoS One*. 23;6(2):e16579.
27. Oron, G., R. Armon, R. Mandelbaum, Y. Manor, C. Campos, L. Gillerman, M. Salgot, C. Gerba, I. Klein, and C. Enriquez. 2001. Secondary wastewater disposal for crop irrigation with minimal risks. *Water Sci Technol*. 43(10):139-46.
28. Ottosson, J. and T.A. Stenström. 2003. Fecal contamination of greywater and associated microbial risks. *Water Res*. 37(3):645-55.
29. Ottosson, J. and T.A. Stenström. 2003. Growth and reduction of microorganisms in sediments collected from a greywater treatment system. *Lett Appl Microbiol*. 36(3):168-72. Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water.
30. Rijal, G., C. Petropoulou, J.K. Tolson, M. DeFlaun, C. Gerba, R. Gore, T. Glymph, T. Granato, C. O'Connor, L. Kollias, and R. Lanyon. 2009. Dry and wet weather microbial characterization of the Chicago area waterway system. *Water Sci Technol*.;60(7):1847-55.
31. Ruiz N, J.E. Marion, R.D. Miles, and R.B. Barnett. 1987. Nutritive value of new cultivars of triticale and wheat for broiler chick diets. *Poult Sci*. 1987 Jan;66(1):90-7.
32. Salvadori, M.I., J.M. Sontrop, A.X. Garg, L.M. Moist, R.S. Suri, W.F. Clark WF. 2009. Factors that led to the Walkerton tragedy. *Kidney Int Suppl*. (112):S33-4

33. Savageau, M.A. 1998. Demand theory of gene regulation. II. Quantitative application to the lactose and maltose operons of *Escherichia coli*. *Genetics* 149:1677-1691 1998
34. Sears, H.J., H. Janes, R. Saloum, I. Brownlee, and L.F. Lamoreaux. 1956. Persistence of individual strains of *Escherichia coli* in man and dog under varying conditions. *J Bacteriol.* 71(3):370-2.
35. Söderström, A., P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. Blide Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G. Allestam, I Hedenström, L. Ledet Muller, and Y. Andersson. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog Dis.* 5(3):339-49
36. Solomon, E.B., C.J. Potenski, and K.R. Matthews. 2002. Effect of irrigation method on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. *J Food Prot.* 65(4):673-6
37. Standing, T.A., E. du Plessis E, S. Duvenage, L. Korsten. 2013. Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. *J Water Health.* 11(2):210-23. doi: 10.2166/wh.2013.164

38. Temple, K.L., A.K. Camper, and G.A. McFeters. 1980. Survival of two enterobacteria in feces buried in soil under field conditions. *Appl Environ Microbiol.* 40(4):794-7
39. The United States Department of Agriculture National Agricultural Statistics Service. (NASS) 2008. Available at:
https://www.nass.usda.gov/Publications/Ag_Statistics/2008/2008.pdf
40. The United States Food and Drug Administration Center for Food Safety and Applied Nutrition. (CFSAN FDA), 2007
<https://www.fda.gov/downloads/ICECI/UCM090298.pdf>
41. Wheeler, C., T.M. Vogt, G.L. Armstrong, G. Vaughan G, A. Weltman, O.V. Nainan, V. Dato, G. Xia, K. Waller, J. Amon, T.M Lee, A. Highbaugh-Battle, C. Hembree, S. Evenson , M.A. Ruta, I.T. Williams, A.E. Fiore and B.P. Bell. 2005. An outbreak of hepatitis A associated with green onions. *N Engl J Med.* 353(9):890-7.
42. Winfield M.D. and E.A. Groisman. 2003 Role of nonhost environments in the lifestyles of Salmonella and Escherichia coli. *Appl Environ Microbiol.* 69(7):3687-94.
43. Won, G., P.J. Schlegel, J.M. Schrock, and J.T. LeJeune. 2013. Absence of direct association between coliforms and Escherichia coli in irrigation water and on produce. *J Food Prot.* 76(6):959-66.

Chapter 3

USE OF ZERO-VALENT IRON FILTRATION TO ENHANCE IRRIGATION WATER QUALITY

3.1 Abstract

Contaminated irrigation water has previously been identified or implicated as the source for previous multistate produce outbreaks. Zero-valent iron (ZVI) has been shown to be effective in the remediation of groundwater and the reduction of bacterial contamination in irrigation water. Due to the risk of fecal contamination and potential reduction in the microbiological quality of irrigation water, some surface water may not meet FDA water quality standards as outlined in the FDA Produce Rule. Mitigation strategies should be applied to questionable water sources prior to use on raw agriculture commodities. This study assessed water filtration using zero-valent iron with sand and sand alone to reduce *E. coli* and *S. Newport* in water with a relatively high organic load used to irrigate field-grown tomatoes. Filtration resulted in reductions of 2.04 ± 0.55 and 4.75 ± 0.13 log CFU/100 ml of *E. coli* and *S. Newport*, respectively. Tomato fruits collected from experimental field plots irrigated with unfiltered water (2.23 ± 0.46 log CFU/100 ml) contained significantly higher bacterial counts than fruits irrigated with sand-filtered water (1.24 ± 1.40 log CFU/100 ml) and ZVI-filtered water (0.65 ± 1.05 log CFU/100 ml) ($P < 0.05$). Following analysis of the contents of both columns, *E. coli* recovery was significantly lower in all four ZVI sections when compared to the sand only column ($P < 0.05$). This study builds on the growing body of literature to support the potential use of ZVI in agriculture as an alternative treatment for irrigation water containing high organic loads. Filtration units containing ZVI may be a useful to ensure agriculture water meets current regulatory standards.

3.2 Introduction

It is estimated that approximately 70% of the world's fresh water resources are used for irrigation of crops, which is three times the amount of water that was used for agriculture 50 years ago (Global Agriculture, 2016). By the year 2050, it is expected that the amount of water used for irrigation will increase by another 19% (Global Agriculture, 2016). Therefore, access to water sources of high quality for irrigation of crops is important in order to reduce any potential public health risks. Produce irrigated with water of poor microbiological quality may be subjected to raised levels of bacteria as well as microbial pathogens (Benjamin *et al.* 2013, Won *et al.* 2013, Park *et al.* 2012, Castro-Rosas *et al.* 2012, Standing *et al.* 2013, Erickson, 2012). However, many fruit and vegetable producers have limited options when selecting quality water supply sources. Rivers, streams, canals and other types of surface water typically used as sources for irrigation water are open to wildlife and the environment and therefore; potential contamination. In addition, water may be exposed to manure run-off or direct contamination by animal intrusion. Due to varying levels of risk in water sources, the FDA Food Safety Modernization Act (FSMA)-Produce Safety Rule will require 4 initial ground water samples to be tested annually and 20 initial surface water samples over 2-4 years (FDA, 2015). These circumstances combined with the basic understanding that water is becoming a limited resource and vital to the production of fruits and vegetables (Odumeru, 2013) highlight the need for the development of effective "hurdle" technologies for the improvement agricultural water quality.

Farm management practices involving water (such irrigation and crop protection applications) have previously been identified as potential sources of food-surface contamination (Harris *et al.* 2012). One common farm practice involves the spreading of manure on agricultural fields under animal manure management systems in the U.S., despite known contamination risk such as manure run-off (Harris *et al.* 2013, Ziemer *et al.* 2010). The intrusion of wildlife and farm animals, who often utilize the same water sources used for irrigation, likely perpetuates the spread of pathogens into the environment (Gorski *et al.* 2011). Biosand Filtration (BsF), Ozonation, oxidizing sanitizers, and UV treatments are applied to water used in post-harvest washing practices to reduce cross-contamination with human pathogens (Wang *et al.* 2007). While proven effective at reducing microbial populations, chlorine-based treatments are most commonly used. Ozone and UV applications work also but are costly and lack practicality for on-farm use.

Annually in the U.S., there are 48 million illnesses associated with the consumption of contaminated food products, a growing portion of these may be fresh produce (CDC, 2011). Specifically, *Salmonella* spp. have been identified as the bacterial pathogen most often linked to these infections (CDC, 2011). *Salmonella* is an enteric organism that is recognized for extended survival outside its primary host (animals), such as in soil and water used for fruit and vegetable production (Fish and Pettibone 1995, Winfried and Groisman, 2003; Barak and Liang, 2008; Hanning et al 2009; Garcia et al. 2010; Bell et al., 2015). *Salmonella* may contaminate food crops directly or indirectly through manure or soil amendments, irrigation water, dust, or

poor farm worker hygiene (Haley et al 2009 and Wacheck et al 2010). More importantly, when produce wash water is poorly disinfected, cross-contamination can occur between *Salmonella* and produce commodities (Doyle and Erickson, 2008; Hanning, 2009)

The addition of zero-valent iron (ZVI) to Bio-sand filtration (BSF) is a potential alternative for the mitigation of contaminated irrigation water. ZVI, a waste product of iron processing, continuously oxidizes in water through reactions with dissolved oxygen and protons to form amorphous iron hydroxides (Phillips *et al.* 2010). Iron hydroxides are subsequently converted to more stable oxides and hydroxides. As the water moves through the ZVI matrix, these reactions can help absorb viruses and other negatively-charged microorganisms through electrostatic interactions (Phillips *et al.* 2010). A ZVI filtration study conducted previously by Ingram *et al.* (2012), demonstrated a 6-log CFU 100ml⁻¹ reduction of *E. coli* O157:H12 from irrigation water using ZVI, which was 0.49 log CFU100ml⁻¹ higher compared to sand filtration ($P<0.05$).

The use of *E. coli* as an indicator of water quality safety originated 100 year ago when US Public Health Service (PHS) set microbial limits for safe drinking water (Pontius et al 2005). Growers in California worked quickly to design the California Leafy Greens Marketing Agreement (LGMA) following the September 2006 multistate outbreak in bagged spinach. This document, based on Environmental Protection Agency (EPA) recreational water standards, set the threshold at 126 MPN 100 ml⁻¹ Geometric Mean which is used for *E. coli* levels in irrigation water

application. Consistent with LGMA standards, generic *E. coli* remains the recommended indicator organism for evaluation of water used during irrigation and in production facilities. The FDA Produce Safety Rule, within the Food Safety Modernization Act, allows growers to utilize water sources that may not have originally met the *E. coli* metrics after periodic testing and record-keeping over time. FDA will not plan to mandate any prescribed methods for treating water that is above the microbiological threshold. However, adhering to the FDA Produce Rule, growers must remediate water sources to lower generic *E. coli* levels to those stated in the final regulations. Unfortunately, guidance documents do not recommend effective technologies for growers to improve water quality. Of the current treatments available, including well water shocking, and treatment with antimicrobials or chemicals, most are costly or not approved for use on raw produce at pre-harvest. Other solutions like building berms and finding alternative sources of water are time-consuming. Growers and regulators are in need of a cost effective and robust innovative method to treat irrigation water. ZVI-enhanced filtration is a potential means to improve agricultural water quality. The current study builds on the growing body of literature to support the potential use of ZVI in agriculture as an alternative treatment for irrigation water of questionable microbial quality.

3.3 Materials and Methods

3.3.1 Preparation of Manure Water Contaminated with *E.coli*

Fresh manure samples (500g) were collected at random from the University of Delaware Dairy Farm. Manure (111g) was added to 11L of deionized water. The sample was agitated for 2 minutes, allowed to sit for approximately ten minutes at room temperature, agitated and again for 2 min. Serial dilutions of manure water were performed in PBS for enumeration of *E.coli* on Tryptone Bile X-Glucuronide agar (TBX, Oxoid, UK) and incubated at 37°C for 24 hrs. A prepared 11L water sample typically contained ~6 log CFU 100 ml⁻¹ *E. coli*.

3.3.2 *Salmonella* Culture Preparation for Inoculated Pond Water.

A rifampicin (Fisher Bioreagents, Fair Lawn, NJ, USA) resistant strain of *S. Newport* (MDD 314), stored in 1.8 ml Lactose broth (Difco, Becton, Dickinson, Sparks, MD, USA) containing 20% glycerol at -80 °C, was grown in tryptic soy broth with Rifampicin (TSBR; 80 µg ml⁻¹; Difco, Becton, Dickinson, Sparks, MD, USA). *S. Newport* was originally isolated from the state of Virginia following a tomato outbreak and generously donated by Dr. Michelle Danylunk at the University of Florida (Gainesville, FL, USA). Surface water samples (125L) were collected from a surface-water pond (Dover, DE, USA) within areas of active irrigation during the early spring of 2014. Pond water was held at 4°C prior to being used. One isolated colony of MDD314 was subsequently inoculated into 700 ml TSBR and incubated at 35 ± 2 °C for 18 h, centrifuged at 3000 rpm for 10 min and the pellet washed and resuspended in 0.1% peptone water then repeated (PW, Difco, Becton, Dickinson,

Sparks, MD, USA). The resuspended bacterial culture (110 ml) was diluted 1:100 in pond water and mixed manually for 2 minutes in 20-L carboys (Nalgene, Rochester, NY, USA) before filtration, for a final concentration of c. $7 \log \text{CFU } 100 \text{ ml}^{-1}$ *Salmonella* Newport. Serial dilutions of inoculated pond water were enumerated on TSAR (TSAR; $80 \mu\text{g ml}^{-1}$; Difco, Becton, Dickinson, Sparks, MD, USA) and incubated at $35 \pm 2 \text{ }^\circ\text{C}$ for $24 \pm 2 \text{ h}$.

3.3.3 Filter Preparation.

Commercial HydrAid Biosand filters (Cascade Engineering, Grand Rapids, MI, USA) were built as described in the preparation manual and as described previously by Ingram et al. (2012). In brief, each 20-L column contained gravel (6mm-12mm), filtration gravel (0.7mm-6mm) and 45.4 kg of filtration sand ($\leq 0.7\text{mm}$). Filters are 0.77 m high with a diameter of 0.14 m. Filter casing weight is was 3.6-kg empty and 63.5-kg when filled with sand and gravel. Filters were modified to contain either a filtration sand layer only or a combination of zero-valent iron (ZVI, Peerless Metal Powder and Abrasives, Detroit, MI, USA) and sand (purchased at a local hardware store in Newark, DE, USA) at a (1:1) ratio by weight. Following initial setup, deionized water (11L) was added daily for two weeks before use in field trials.

3.3.4 Filtration of Water Samples.

Manure water (11L) was added twice to each filter followed by 11L samples of deionized water. The rate of each 11L flush ranged from 1.5- 4.1 L/hr. Coliform and *E. coli* concentrations were assessed in pre- and post-filtered water samples on TBX agar.

Water was preliminarily monitored for dissolved solids, *E. coli*, total coliforms, and *Salmonella* spp. Water parameters were determined using a portable environmental monitoring system (Sper Scientific, Scottsdale, AZ, USA) and a complete evaluation was performed by the University of Delaware Soil Testing Laboratory. Pond water was inoculated with *S. Newport* to a final concentration of ca. $6 \log \text{CFU } 100 \text{ ml}^{-1}$ *Salmonella* Newport prior to filtration. *S. Newport* was enumerated on TSAR and enriched as necessary following a modified BAM procedure using RV broth (FDA, 1998). For *Salmonella* enrichment, an equal volume (10 ml) of 2X lactose broth was added to the sample and incubated at $35 \pm 2^\circ\text{C}$ for 24 h. After 12-18 h incubation, 1 ml of sample was added to 9 ml of tetrathionate (TT; Difco, Becton Dickinson, Sparks, MD) broth and 0.1 ml of enrichment was added to 9.9 ml of Rappaport-Vassiliadis (RV; Difco, Becton Dickinson, Sparks, MD). TT broth and RV broth tubes were incubated at $35 \pm 2^\circ\text{C}$ for 24 h and $42 \pm 2^\circ\text{C}$ for 48 h, respectively. Following incubation, 10 μl from each broth was streaked onto XLD Agar supplemented with rifampicin (XLDR; 80 $\mu\text{g/ml}$; Difco, Becton, Dickinson, Sparks, MD). XLDR was incubated at $35 \pm 2^\circ\text{C}$ for 24 h. When enrichments were positive, populations were recorded as 0.3 log CFU/ml (limit of detection).

Water was gravity-fed over ZVI and sand filtration units with the addition of 11L at a time and repeated collection of two 50 ml samples following the filtration of each liter were collected. Samples were 10-fold diluted in 0.1% PW, then plated (100 ul), in duplicate, on TBX and TSA-R for *E. coli* and *Salmonella* Newport, respectively. In addition, the water samples were screened using Colilert according to the manufacturer's instructions with Quanti-Tray/2000 (IDEXX, Westbrook, ME, USA) to detect coliforms and *E. coli* or filtered through 0.45-mm MicroCheck II beverage monitors (Pall Corp., Ann Arbor, MI, USA) placed in vacuum unit. Resulting filters were aseptically removed using sterile forceps and placed on TBX and TSA-R.

3.3.5 Plant Preparation

Tomato (BHN-602) seeds (Johnny Selected Seeds, Winslow, ME, USA) were sown in 2401 seed trays (Dillen Products, Middlefield, OH, USA) containing ProMix soil (Premier Horticultural Inc. Quakertown, PA, USA). Seed trays were placed in the UDBG misting room (22-24°C, 85% humidity) until germination. Upon germination, plants were moved out to the UDBG main greenhouse (28-30°C, 60% humidity). Tomatoes were transplanted into 4" square traditional thin wall pots (Dillen Products, Middlefield, OH, USA) at 3 weeks. Plants were fertilized using Peter's Fertilizer (21-5-20) at a concentration of 200ppm nitrogen. A Dosatron (Dosatron International, Clearwater, FL, USA) set at a rate of 1:128 was used to apply fertilizer once per week while in the green house. At 6 weeks, seedlings were moved outside where they were

hardened for 1 week. Following the hardening period, they were transplanted into plastic-culture (Rain-Flo, East Earl, PA, USA). Irrigation was performed using Drip Tape (Rain-Flo, East Earl, PA, USA) until full maturity. Tomatoes were grown in single rows with stakes (Honduran Pine Stakes) every 4' and plants evenly spaced at 18" apart.

3.3.6 Plant Inoculation

Fifteen-week old plants (180 total, 12 groups of 15 plants with 4 treatments in triplicate) were inoculated with (1) ZVI- filtered water, (2) sand-filtered water, (3) unfiltered manure water or (4) sterile deionized water. Plants were inoculated with 250 ml inoculum per plant, saturating top leaves and fruits. Each plant was inoculated individually using a sterilized graduated cylinder.

3.3.7 Sampling and *E. coli* Enumeration

Samples were collected on day 0 pre- and post-inoculation, and on days 1, 3, 5 post-inoculation. Samples were composites of four tomatoes taken from four random plants within each plot. Fruit samples (4 tomatoes of 500-600-g total from 4 plants per plot) were weighed upon collection and placed into 3.78-l Ziploc bags (SE Johnson, Chicago, IL, USA) with phosphate buffered saline to a 1:1 w:v ratio, based on tomato weight. Each sample was chosen at random using a random number selection system for each plot. After 1 minute of shaking by hand in Phosphate Buffer Saline (PBS,

Sigma Aldrich, St. Louis, MO, USA), samples were serially diluted in 9ml of Phosphate Buffer Saline (PBS, Sigma Aldrich, St. Louis, MO, USA) and bacteria enumerated on TBX in duplicate after incubation at 37°C for 24hrs.

3.3.8 Column Deconstruction

The ZVI and sand filtration columns were disassembled, drained, and filter contents were sectioned as shown in (Figure 1). Wet-packed filter contents were aseptically removed using sterile spoons. The upper 0.125 m portion of the filter was removed first, then each of the four sections (0.125 m of filter contents), were collected to form a 150-g composite sample of sand or zvi-sand mixture and transferred into 3.78-l Ziploc bags and three 25-g samples were placed in 80-ml bags to which 250-ml of 0.1% PBS was added. Samples were hand massaged for 2 minutes. Serial dilutions were performed using 0.1% PW and 0.1-ml was plated on TBX in duplicate and incubated 24 h at 37°C.

3.3.9 Water Analysis

Sample pH (1:1 v:v) was measured using an Accumet pH meter model AB15 and a SymPHony pH electrode (Fisher Scientific, Pittsburgh, PA). Ammonium-N and nitrate-N were measured colorimetrically using a Bran&Luebbe AutoAnalyzer 3 (Bran&Luebbe, Buffalo Grove, IL). Ortho-phosphate phosphorus was measured

colorimetrically by the Modified Murphy-Reilly method using a Sequoia-Turner Spectrophotometer Model 340 (Sequoia-Turner Corporation, Mountain View, CA). Total carbon, total inorganic carbon and total bound nitrogen were measured by direct combustion and total organic carbon was calculated by difference using an Elementar Vario-Cube TOC Analyzer (Elementar Americas, Mt. Holly, NJ).

Statistical analysis. All filtration experiments were completed in duplicate (8 samples per pore volume). The means of the data for *S. Newport* and *E. coli* were calculated for the water samples. The statistical difference between the populations of bacteria in the different filtration column and experimental plots was evaluated using least-squares means. Multifactor analysis of variance tests for treatment and organism interactions within each column and experimental plot was calculated. All data was processed using JMP software (version 11.0; SAS Institute Inc., Cary, NC, USA). The alpha value was set at 0.05.

3.4 Results and Discussion

Since populations of both, *E. coli* and *S. Newport* have been associated with agricultural water (CFR, 2007). To determine the survival of bacterial populations in agricultural water following filtration, *E. coli* and *S. Newport* were used within the inoculated water. *S. Newport*, has been associated with produce outbreaks, including those that have occurred in the Mid-Atlantic and Delmarva areas (Bell *et al.* 2015) and as mentioned above, *E. coli* populations are used as the standard to measure fecal contamination in recreational drinking water as well as for FSMA regulatory

thresholds. In this study, generic *E. coli* and *S. Newport* inoculated in agricultural water were filtered through gravity-fed columns at a rate of 800ml min⁻¹, and resulting bacterial populations were quantified (Table 1).

ZVI has been previously shown more effective than sand filters to significantly decontaminate irrigation water containing *E. coli* O157:H7 surrogate, reducing *E. coli* O157:H12 by 1.7 log CFU 100 ml⁻¹, which was similar to the 1.5 log CFU 100ml⁻¹ reduction found in this study (Ingram *et al.* 2012). In our study, starting concentrations in water before filtration averaged 6.17 and 7.32 log CFU 100ml⁻¹ for *E. coli* and *S. Newport*, respectively. One pore volume (11 L) flowed through the filter at a time. *S. Newport* populations found in ZVI-filtered water were below the detection limit (0.80 log CFU 100ml⁻¹) compared with populations recover from Sand-filtered water (4.66 log CFU 100ml⁻¹), which were both significantly reduced from *S. Newport* inoculated unfiltered pond water (7.32 log CFU 100ml⁻¹). Similarly, initial *E. coli* concentrations from both ZVI-filtered and Sand-filtered water were reduced significantly using both treatments ($P < 0.05$). *E. coli* populations from ZVI-filtered water (4.44 log CFU 100ml⁻¹) and Sand-filtered water (5.30 log CFU 100ml⁻¹) were significantly lower ($P < 0.05$) in comparison with unfiltered water containing dairy manure (6.17 log CFU 100 ml⁻¹). However, there was no significant statistical difference between sand and ZVI treatments when assessing *E. coli* populations in filtered water following the first pore volume. After the second 11L pore volume was filtered, *E. coli* populations in ZVI-filtered water (4.13 log CFU 100 ml⁻¹) were significantly lower in comparison with populations in sand-filtered water (4.61 log CFU 100 ml⁻¹) ($P < 0.05$). Although,

enumerated *E. coli* levels were not below FSMA standards (2.1 log CFU 100 ml⁻¹ or 126 CFU ml⁻¹). These results indicate that filtration through columns containing a mixture of sand and ZVI can enhance removal of bacterial pathogens compared to Sand-only columns. It is also important to note that concentrations of *E. coli* in irrigation water are not typically this high and were simulating a “worst case scenario” event

Following inoculation of tomato plots, *E. coli* populations on tomato fruits recovered after plants were irrigated with unfiltered water or with water filtered through sand only or sand and ZVI filter columns as depicted in Table 2. Since *E. coli* populations are commonly isolated from tomato production environments, 12 composite fruit samples were collected prior to inoculation to determine potential background populations. In all baseline samples from both 2013 and 2014 trials, no *E. coli* was present on tomatoes 24 h before inoculation (data not shown). These data showed *E. coli* populations on tomato fruit collected immediately following inoculation (day 0) with ZVI-filtered water averaged 0.65 log CFU ml⁻¹. Tomato fruit collected from plants irrigated with sand-filtered water contained averaged 1.24 log CFU ml⁻¹, while fruits irrigated with unfiltered water contained an average of 2.23 log CFU ml⁻¹. On day 0, fruits from plants irrigated with ZVI or sand filtered water had significantly lower *E. coli* populations than those irrigated with unfiltered water ($P < 0.05$). Interestingly, on days 1,3, and 5, daily average *E. coli* populations from both sand and ZVI irrigated plots remained below 1 log CFU ml⁻¹, while unfiltered plots

reduced to daily averages ranging from 1.06 to 1.18 log CFU ml⁻¹. There was no significant difference comparing treatments and unfiltered plots on days 1,3 and 5.

It is important to note that in both the 2013 and 2014 trials, a large number of samples, including unfiltered manure water, had non-detectable levels of *E. coli*. The bacterial levels found in these field trials were consistent with work performed previously by this group using smaller-scaled filtration units and spinach tissue to assess the effectiveness of ZVI to reduce *E. coli* O157:H7 in irrigation water (Ingram *et al.* 2012). Both previous and the current studies reported generic and pathogenic *E. coli* levels on fruit and vegetable tissues to be below 1 log CFU ml⁻¹, respectively (Ingram *et al.* 2012). While future work still needs to be performed to understand the mechanism in which ZVI inactivates microorganisms, these results suggest the use of ZVI to reduce bacterial populations in agricultural water is encouraging.

Filter deconstruction was performed following the filtration studies to determine if residual *E. coli* populations remained in the filter substrates from both sand and ZVI columns. Filtration sand and ZVI particles were analyzed for populations of *E. coli* as columns based on location in columns from top to bottom and are depicted in Figure 1. The inoculated columns were divided into four sections (TOP, MID, MID2, BOT). *E. coli* populations were detected in all sections of both sand and ZVI columns. Interestingly, several samples from the BOT section of the ZVI column did not have detectable *E. coli* present, supporting previous hypothesis that ZVI can inactivate bacteria. Within each section, sand filtration particles

contained significantly higher populations in comparison to ZVI filtration particles ($P < 0.05$). Similar to findings from work reported by Ingram et al (2012), the bottom most sections of the filter columns contained significantly lower *E. coli* counts relative to upper portions ($P < 0.05$), suggesting *E. coli* populations maybe more localized to the top of the column than the bottom, which may help trap *E. coli* from being collected before use as irrigation water.

The work presented here is the first of its kind to use *E. coli* naturally present in dairy manure to evaluate the usefulness of ZVI filtration systems. While similar studies by this group have presented ZVI as an intervention step to effectively reduce microbial concentrations in irrigation water, these results stand only as the first to use ZVI-filtered water to irrigate tomatoes. Studies assessing the dynamic environment of agriculture water described here may help understand agricultural water and the challenges that food safety experts, companies and regulators face when evaluating irrigation water for the transmission of fecal contamination on fresh tomatoes. In conclusion, ZVI may serve as a reasonable means to reduce generic *E. coli* and pathogenic *S. Newport* levels in irrigation water.

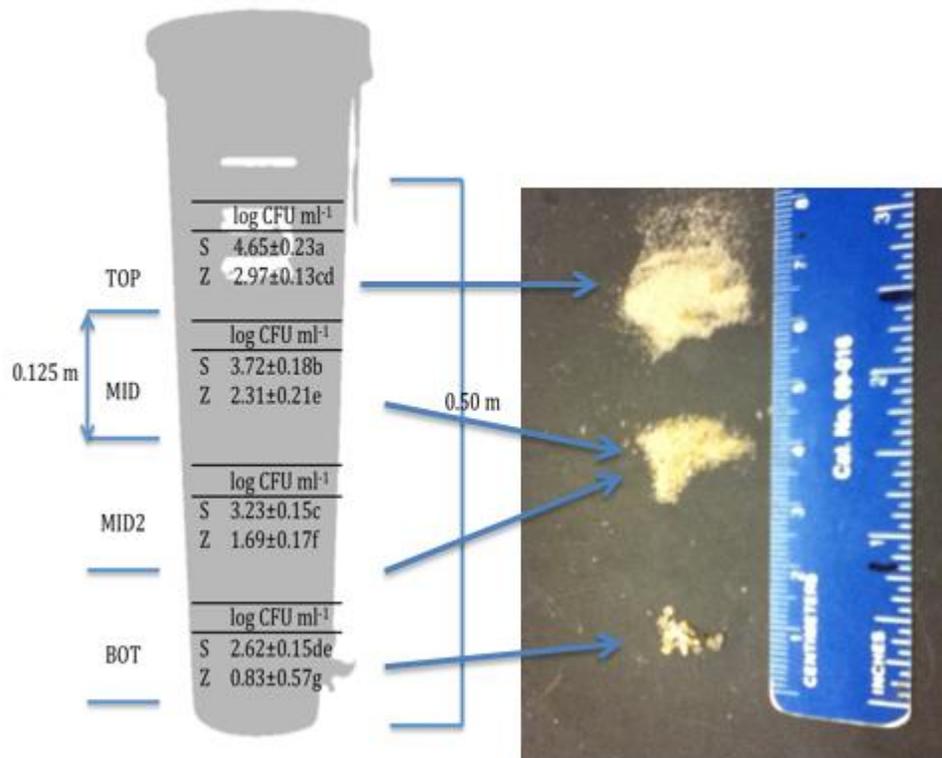


Figure 13. Generic *E. coli* recovery (log CFU ml⁻¹) within either sand or sand-ZVI filter column following treatment of manure water. Deconstructed columns were sectioned in four equal (TOP, MID, MID2, BOT) regions measuring 0.125 m. Within whole model, daily means across both filters followed by different letters (a,b,c,d,e,f,g) are significantly different ($P<0.05$). Super fine sand was placed in TOP section, while MID and MID2 contained fine sand. The BOT section was composed on level two media which was slight larger in grain size than both upper regions.

Table 4. Populations of generic *E. coli* and *S. Newport* in filtered water.

Liter	Population (log CFU/100ml) of <i>Salmonella</i> serovar Newport and <i>E. coli</i> in Inoculated water collected in carboys			
	Treatment (n=10)			
	Sand		Sand-ZVI	
	<i>E. coli</i>	<i>Salmonella</i>	<i>E. coli</i>	<i>Salmonella</i>
Initial Inoculum	6.17±0.18x#b*	7.32±0.74wa	6.17±0.18xb	7.32±0.74wa
First 11L	5.30±0.13yc	4.66±0.13yd	4.44±0.37yde	ND
Second 11L	4.61±0.28yd	5.69±0.23xc	4.13±0.55ze	2.57±0.13zf

#Within inoculum, daily means in the same column followed by different letters (x,y,z) are significantly different ($P < 0.05$)

*Within whole model, daily means across all treatments followed by different letters (a,b,c,d) are significantly different ($P < 0.05$)

Table 2. Populations of generic *E. coli* on tomato fruit following irrigation with filtered water from either no treatment, sand-ZVI or sand filtration columns

Day	Population (log CFU/ml) of <i>E. coli</i> collected from tomato fruit		
	Treatment (n=9)		
	Control	Sand	Sand-ZVI
0	2.23±0.46a#w#	1.24±1.40ax	0.65±1.05axyz
1	1.18±1.41axy	0.38±0.25bz	0.35±0.16az
3	1.04±0.66axy	0.52±0.66abyz	ND
5	1.06±1.23axyz	0.54±0.57abyz	0.57±0.81axyz

*Within treatment, daily means in the same column followed by different letters (a,b) are significantly different ($P < 0.05$)

#Whole model, daily means across all treatments followed by different letters (w,x,y,z) are significantly different ($P < 0.05$)

REFERENCES

1. Barak, J. D. and A. S. Liang, 2008. Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *Plos ONE* 3:e1657.
2. Bell R. L, J. Zheng, E. Burrows, S. Allard, C.Y Wang, M.W. Allard, S. Rideout and E.W. Brown. 2015. Ecological prevalence, genetic diversity, and epidemiological aspects of *Salmonella* isolated from tomato agricultural regions in the Virginia Easter Shore. *Front. Microbiol.* 6:415.
3. Benjamin L., E. R. Atwill, *et al.* 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the central California coast. *Intl. J. Food Microbiol.* 165:65-76.
4. Castro-Rosas, J., J.F. Cerna-Cortés, E. Méndez-Reyes, D. Lopez-Hernandez, C. A. Gómez-Aldapa, T. Estrada-Garcia. 2012. Presence of fecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *Int J Food Microbiol.* 2012 May 15;156(2):176-80. doi: 10.1016/j.ijfoodmicro.2012.03.025. Epub 2012 Mar 30. PMID: 22507628

5. Derevianko, S., P. Chiu, Y. Jin, M. Sharma, and K.E. Kniel. 2008 not yet published. Effect of zero-valent iron on removal of *Escherichia coli* O157:H7 from water.
6. Doyle, M. P., and M. C. Erickson. 2008. Sumer meeting 2007- the problems with fresh produce; an overview. *J. Appl. Microbiol.* 105, 317-330.
7. Erickson, M. 2012. Prevalence of enteric pathogens/surrogates in environmental water sources [table]. Available at <http://www.ugacfs.org/producesafety/Word_documents/PathogenPrevalenceInWater.doc>. Accessed 24 March 2016.
8. Fish, J.T. and G. W. Pettibone. 1995. Influence of freshwater sediment on the survival *Escherichia coli* and *Salmonella* sp. as measured by three methods of enumeration. *Lett. Appl. Microbiol.* 20, 277-281.
9. Garcia, R., J. Baelum, L. Freslund, P. Santorum, and C.S. Jacobsen. 2010. Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurim and expression of *invA* in soil and manure-amended soil. *Appl. Environ. Microbiol.* 76, 5025-5031.
10. Global Agriculture. 2016. Agriculture at a Crossroads: Findings and recommendations for future farming – water. Accessed at: <www.globalagriculture.org/report-topics/water.html>. Accessed on 17 Aug. 2016.

11. Gorski, L., C.T Parker, A. Liang, M.B. Cooley, M.T. Jay-Russell, et al. 2011
Prevalence, distribution, and diversity of *Salmonella enterica* in a major
produce region of California. *App and Environ Microbiol.* 77: 2734–2748.
12. Haley B. J., D. J. Cole, and E. K. Lipp. 2009. Distribution, diversity, and
seasonality of waterborne *Salmonella* in a rural watershed. *Appl. Environ.*
Microbiol. 75:1248
13. Hanning, I. B., J.D. Nutt, and S. C. Ricke. 2009. Salmonellosis outbreaks in
the United States due to fresh produce: sources and potential intervention
measures. *Foodborne Pathog. Dis.* 6, 635-648.
14. Harris L. J., J. Bender, E.A. Bihn, T. Blessington, M.D. Danyluk, P. Delaquis,
L. Goodridge, A.M. Ibekwe, S. Ilic, K.E. Kniel, J.T. Lejeune, D.W. Schaffner,
D. Stoeckel, and T.V. Suslow. 2012. A framework for developing research
protocols for evaluation of microbial hazards and controls during production
that pertain to the quality of agricultural water contacting fresh produce that
may be consumed raw. *J. Food Prot.* 75:2251-2273.
15. Harris L.J., E.D. Berry, T. Blessington, M. Erickson, M. Jay-Russell, X. Jiang,
K. Killinger, F.C. Michel, P. Millner, K. Schneider, M. Sharma, T.V. Suslow,
L. Wang , and R.W. Worobo. 2013. A framework for developing research
protocols for evaluation of microbial hazards and controls during production
that pertain to the application of untreated soil amendments of animal origin on
land used to grow produce that may be consumed raw. *J Food Prot.*
76(6):1062-84.

16. Ingram D.T., M.T. Callahan, S. Ferguson, D.G. Hoover, P.C. Chiu, D.R. Shelton, P.D. Millner, M.J. Camp, J.R. Patel, K.E. Kniel, and M. Sharma. 2012. Use of zero-valent iron biosand filters to reduce *Escherichia coli* O157:H12 in irrigation water applied to spinach plants in a field setting. *J. Appl Microbiol.* 112:551-560.
17. Odumeru B., Call to Action- Let's put water on a HACCP plan. International Association of Food Protection 2013. RT-11.
18. Park S., S. Navratil, A. Gregory, A. Bauer, I. Srinath, M. Jun, B. Szonyi, K. Nightingale, J. Anciso, and R. Ivanek. 2013. Generic *Escherichia coli* contamination of spinach at the preharvest stage: effects of farm management and environmental factors. *Appl Environ Microbiol.* 79:4347-4358.
19. Phillips D.H., T. Van Nooten, L. Bastiaens, M.I. Russell, K. Dickson, S. Plant, J.M. Ahad, T. Newton, T. Elliot, and R.M. Kalin. 2010. Ten year performance evaluation of a field-scale zero-valent iron permeable reactive barrier installed to remediate trichloroethene contaminated groundwater. *Environ. Sci. Technol.* 44:3861-3869.
20. Pontius, F. W. History of the Safe Drinking Water Act (SDWA), in Drinking Water Regulation and Health (ed F. W. Pontius), John Wiley & Sons, Inc., Hoboken, NJ, USA. 2005. doi: 10.1002/0471721999.ch4
21. Söderström A., P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. Blide Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G.

- Allestam, I. Hedenström, L. Ledet Muller, and Y. Andersson. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog. Dis.* 5:339-349.
22. Standing, T.A., E. du Plessis, S. Duvenage, and L. Korsten. 2013. Internalization potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *Enterica* serovar *Typhimurium* and *Staphylococcus aureus* in lettuce seedlings and mature plants. *J Water Health.* Jun;11(2):210-23. doi: 10.2166/wh.2013.164. PMID: 23708570
23. The California Leafy Green Marketing Agreement. California Leafy Green Marketing Agreement: Commodity specific food safety guidelines for the production and harvest of lettuce and leafy greens. 2013. Available at: <http://www.caleafygreens.ca.gov/sites/default/files/California%20LGMA%20metrics%2008%2026%2013%20%20Final.pdf>. Accessed 22 November 2013.
24. The Center for Disease Control. 2011. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food-foodborne diseases active surveillance network, 10 U.S. sites 1996-2010. *MMWR Mor. Mortal. Wkly. Rep.* 60, 749-755.
25. The United States Food and Drug Administration Center for Food Safety and Applied Nutrition. (CFSAN FDA), 2007
<https://www.fda.gov/downloads/ICECI/UCM090298.pdf>
26. The U.S Food and Drug Administration. Standards for the growing, harvesting, packing, and holding of produce for human consumption. (2015)

Available at: <https://www.federalregister.gov/articles/2015/11/27/2015-28159/standards-for-the-growing-harvesting-packing-and-holding-of-produce-for-human-consumption>. Accessed 14 March 2016.

27. The U.S Food and Drug Administration. 1998. Bacteriological Analytical Manual, 8th Edition, Revision A, Chapter 4.
28. Wacheck, S., M. Fredriksson-Ahomaa, M. König, A. Stolle, and R Stephan. 2010. Wild boars as an important reservoir for foodborne pathogens. *Foodborne Pathog Dis.*;7(3):307-12.
29. Wang, H., W. Liang, H. Feng, and Y. Luo. 2007. Modeling of the effect of washing solution flow conditions on *Escherichia coli* O157:H7 population reduction on fruit surfaces. *J. Food Prot.* 70:2533-2540.
30. Winfield, M.D., and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol.* 69. 3687-3694.
31. Won G., P.J Schlegel, J.M. Schrock, and J.T. LeJeune. 2013. Absence of direct association between coliforms and *Escherichia coli* in irrigation water and on produce. *J. Food Prot.* 76:959-966.
32. Ziemer C.J., J.M. Bonner, D. Cole, J. Vinjé, V. Constantini, S. Goyal , M. Gramer, R. Mackie, X.J. Meng, G. Myers, and L.J. Saif. 2010. Fate and transport of zoonotic, bacterial, viral, and parasitic pathogens during swine manure treatment, storage, and land application. *J Anim Sci.* 88:E84-94.

Chapter 4

THE FATE OF SIX *SALMONELLA* AND FIVE *E. COLI* SEROTYPES IN DIFFERENT WILD ANIMAL FECES OVER A YEAR

4.1 Abstract

Heightened concerns about wildlife intrusion on the farm and possible pathogen introduction to the food supply have resulted in mandated 30 feet buffer zone between grazing animals and crops in the Leafy Greens Marketing Agreement (LGMA). However, *Salmonella* and *E. coli* transmission from animals continues to bear risks of contamination of fresh produce. Cattle and wild animal feces were obtained from different sources in California, Delaware, Florida, and Ohio. The survival of *Salmonella* in animal feces and the resulting transmission of foodborne illness is a static problem for growers. Several studies along with outbreak epidemiological data suggest that wildlife can serve as vectors for pathogenic bacteria, including various strains of *E. coli* O157:H7 where non-composted manure was used or where animals were present. Initial *Salmonella* population ranged from 5.2 to 6.1 log CFU/g in all fecal samples, and increased until day 7 in all types of fecal material with the exception of pig and raccoon. Initial *E. coli* growth was observed in deer, waterfowl and cattle feces with highest concentrations on 15 days post-inoculation (dpi) at 7.50 log CFU/g. This study describes a comparative analysis of the survival of pathogenic *Salmonella* and *E. coli* serotypes in the feces of five different wildlife and agricultural animals over a year long incubation period.

4.2 Introduction

Each year, *Salmonella* is estimated to cause 19,000 hospitalizations and 380 deaths, with annual medical cost estimated at \$365 million per year (Scallan *et al.* 2011). Foodborne related *Salmonella* strains of animal origin are prevalent and may lead to the contamination of fruits and vegetables (Greig *et al.* 2014 and Harris *et al.*, 2003). *Salmonella* was first isolated from vegetables in the 1950s on watermelons (Harris *et al.* 2003). *Salmonella* has been identified in various commodities including melons, mangos, peppers, and tomatoes (Barton *et al.* 2011, Bennett *et al.* 2014, Guan *et al.* 2003, and Hanning *et al.* 2009) Shiga-toxigenic *Escherichia coli* and *Salmonella enterica* spp. are responsible for a majority of the bacterial foodborne illnesses associated with the consumption of produce (CDC, 2011). Risk of contamination during manure application to agricultural commodities consumed raw is well established (Jones *et al.* 1999). Heightened concerns about wildlife intrusion on the farm and possible pathogen introduction to the food supply have resulted in mandated 30 feet buffer zone between grazing animals and crops in the Leafy Greens Marketing Agreement (LGMA) (Hoar *et al.* 2013). Transmission of *Salmonella* spp. from wild animals and livestock represents a main pre-harvest contamination risk.

In the high profile 2006 outbreak, fecal samples collected from cattle and feral pigs were positive for the outbreak strain, identifying another contributing factor to the *E. coli* outbreak (CDC, 2006; CalFERT, 2007). The presence of domestic and wild animals within production areas may introduce enteric pathogens (Cieslack *et al.* 1993;

Jay et al 2007; Jay-Russell 2013). Significantly fluctuations have been noted year-to-year, when fecal contamination originating from different animals is identified (Meays et al. 2006), where one year the majority of contamination was due to avian, deer, and canine was noted. The following year most contamination was attributed to avian, bovine, and rodent species (Meays et al. 2006). Deer, feral pig, fox, rabbit, raccoon, goose and other migrating birds are common wild animals that can be found in production areas, though wildlife is known to change depending on geographical location.

Improved awareness, management strategies, and identification of routes of contamination caused the development and improvement of risk management systems such as Good Agriculture Practices (GAPs). However, foodborne pathogen transmission from animals continues to bear risks of contamination of fresh produce. *E. coli* and *Salmonella* may contaminate food crops directly or indirectly through manure or soil amendments, irrigation water, dust, and poor hygienic farm practices (Haley et al., 2009 and Wacheck et al., 2010). Animal sources of *Salmonella* are well described in the literature, whereas a need remains for focused research studying fecal transmission of *Salmonella* as well as possible interventions to prevent transfer (Greig et al. 2014). Wildlife, such as raccoons, feral pigs, and deer can harbor a complement of known zoonotic disease (Compton et al., 2008, Jay, 2008) and while other pre-harvest sources of contamination exist, often wild or domestic animals are overlooked (Hanning et al., 2009). Similarly, agricultural animals such as sheep, cattle and swine

have previously been identified as reservoirs, which shed the pathogen into the environment (Jones *et al.*, 2008, Galland *et al.*, 2000, Gorski *et al.*, 2011b, 2013). *Salmonella* is known to survive in a diverse range of extra-intestinal environments such as soil, surface water, and composted manure piles (Wilkes *et al.* 2014, Patchanee *et al.* 2010 and Pell *et al.* 1997). The survival of *Salmonella* in animal feces and the resulting transmission of foodborne illness is a static problem for growers. Specifically, wild birds have been identified as a likely reservoir for pathogenic *Salmonella* (Luechtefeld *et al.*, 1980; Keener *et al.*, 2004; Saleha, 2004). Wild birds are easily able to migrate from compost piles to irrigation ponds and production areas. Irrigation sources that previously were not associated with fecal material that become contaminated could increase the transmission of *Salmonella* on fresh produce (Assadian *et al.*, 1999; Garcia *et al.*, 2001). A review of transmission of pathogens via swine manure by Ziemer *et al.* (2010) reported the prevalence of *Salmonella* in fresh or stored swine waste ranged from 7.2%-100% and 5.2%-22%, respectively. Furthermore, a study monitoring the prevalence of pathogenic bacteria among grazing sheep in California found *Salmonella* in 0.8% of all fecal samples and only 0.4% of soil samples (Hoar *et al.*, 2013). These studies suggest that *Salmonella* prevalence varies by animal species. Similarly, *Salmonella* persistence in soil also varies. *Salmonella* was isolated from soil sampled on an almond orchard associated with an outbreak for the 5 years following the outbreak, where animal manure was applied to the orchard (Uesugi *et al.*, 2007).

Wildlife intrusion may be difficult to predict and control. LGMA and the FDA Produce Rule state that areas of wildlife intrusion should be excluded from harvest due to risk of contamination. Previous research has generally supported the 5-ft no-harvest zone currently used as a means of managing produce safety risks concerning domestic and wild animal fecal contamination. Animals, particularly cattle, may serve as a primary host for bacteria such as *E. coli*, before being released into the environment via fecal droppings (Ferens et al 2011). In 2011, an outbreak of *E. coli* O157:H7 in Oregon was linked to local grown strawberries, which were contaminated by deer (Laidler *et al.* 2013). Investigators found 11 deer samples all positive for *E. coli* O157:H7 in 5 different fields (Laidler *et al.* 2013).

It is common practice to spread manure on agricultural fields under animal manure management systems in the U.S. despite known contamination risks (Harris *et al.* 2013, Ziemer *et al.* 2010). The intrusion of wildlife and farm animals, which often share irrigation sources with crops, likely perpetuates the spread in the environment (Gorski *et al.* 2011). The application of manure to field crops used for animal feed may recycle pathogen populations in livestock (Brackett *et al.* 1999). In many areas, growers have difficulties maintaining barriers and buffers, such as those suggested by the LGMA guidelines, which would prevent wildlife and farm animals from entering vegetable plots, surface water, and compost piles on farms (LGMA, 2010). Regardless of the management practices, growers face complex safety challenges throughout the United States.

This study describes a comparative analysis of the survival of pathogenic *Salmonella* serotypes in the feces of seven different wildlife and agricultural animals over a year long incubation period. Differences in bacterial survival are presented here, could be impacted by dietary differences of wildlife used within this experiment, which was coordinated throughout four distinct geographical regions, providing a wider understanding of the survival of *Salmonella*.

4.3 Materials and Methods

4.3.1 Serotypes Cocktail Profile

Six serotypes of *Salmonella* were assessed. All serotypes were originally available in Dr. Danyluk's culture collection. Rifampicin resistant isolates of *Salmonella* serotypes included Typhimurium (MDD14; strain LT2), Montevideo (MDD22; human isolate from tomato linked outbreak), Anatum (MDD33; clinical isolate from tomato outbreak), Javiana (MDD226; ATCC BAA-1593 PA tomato outbreak), Branderup (MDD227; 04E61556 2-99 Roma tomato outbreak), and Newport (MDD314; Envir. tomato outbreak, VA). Five serotypes of STEC were used. All serotypes are available in Dr. Danyluk's culture collection. Rifampicin resistant STEC serotypes include O145 (Clinical isolate, Romaine outbreak), O104 (Clinical isolate, sprout outbreak), O111 (Clinical isolate, apple juice outbreak, NY), O103 (Venison outbreak), and O157 (Clinical isolate, spinach outbreak).

4.3.2 Inoculum Preparation

Rifampicin (Fisher Bioreagents, Fair Lawn, NJ) resistant *E. coli* and *Salmonella*, stored at $-80\text{ }^{\circ}\text{C}$, were grown in tryptic soy agar with rifampicin (TSAR; $80\text{ }\mu\text{g/ml}$; Difco, Becton, Dickinson, Sparks, MD). Agar plates were incubated at $35 \pm 2\text{ }^{\circ}\text{C}$ for $24 \pm 2\text{ h}$. One isolated colony of Rifampicin resistant strains was transferred into tryptic soy broth with rifampicin (TSBR; Difco, Becton, Dickinson, Sparks, MD) and incubated at $35 \pm 2\text{ }^{\circ}\text{C}$ for 18 h. Approximately $10\text{ }\mu\text{l}$ of overnight growth was transferred into an additional tube of TSBR and incubated at $35 \pm 2\text{ }^{\circ}\text{C}$ for 18 h. Following incubation, cells were collected by centrifugation at $3000 \times g$ for 10 min. The supernatant was removed and 10 mL of 0.1% peptone water was vortexed with the pellet to wash cells. Rifampicin resistant cells were washed three times, and resuspended in 5 mL peptone to obtain concentration of cells (10^9 – 10^{10} CFU/mL). Then cells were diluted to a desired concentration before inoculation (10^6 – 10^7 CFU/mL). An appropriate volume of serotypes was mixed to have an approximately equal number of cells from each serotype to achieve the total desired inoculating dose of 10^5 to 10^6 CFU/g feces.

4.3.3 Cattle and Wild Animal Fecal Preparation

Cattle and wild animal feces were obtained from different sources in California, Delaware, Florida, and Ohio. Types of wild animal feces used included swine, goose, deer, and raccoon. Samples were collected and retained in a refrigerator ($4 \pm 2\text{ }^{\circ}\text{C}$) until use. Dry weight and pH of fecal samples were measured.

4.3.4 Cocktail Inoculation and Bacterial Enumeration

Twenty five gram representative samples of each feces type were placed in Whirl Pak stomacher bags (Whirl-Pak bag, Nasco), and 2.5 ml volume of the cocktail inoculum was added to each bag. The inoculated sample was mixed with a stomacher for 4 minutes. Fecal samples (1g) were put into 15 ml conical centrifuge tubes for each time interval and stored at 20°C. Populations were enumerated on days 0, 1, 3, 5, 7, 14, 28 and every 28 days for up to 1 year. Also, fecal samples without inoculation were analyzed on TSAR in different concentrations to observe any background organisms were growing.

At each sampling time, 9 ml 0.1% peptone (Difco, Becton, Dickinson, Sparks, MD) was added into conical tubes each containing 1 g of feces. Samples were vortexed for 2 minutes and dilutions made in 0.1% peptone prior to spread plating onto TSAR with Cycloheximide (50 µg/ml). If a low cell concentration was expected, 0.25 ml was placed on each of 4 plates to increase the detection limit to 10⁻¹ initial concentration. Plates were incubated at 35 ± 2°C for 24 h.

4.3.5 STEC and *Salmonella* Enrichments

For *Salmonella* enrichment, an equal volume (10 ml) of double strength lactose broth was added to the sample and incubated at 35 ± 2°C for 24 h. After 12-18 hr. incubation, 1 ml of sample was added to 9 ml of tetrathionate (TT; Difco, Becton Dickinson, Sparks, MD) broth and 0.1 ml of enrichment was added to 9.9 ml of Rappaport-Vassiliadis (RV; Difco, Becton Dickinson, Sparks, MD). TT broth and RV

broth tubes were incubated at $35 \pm 2^\circ\text{C}$ for 24 h and $42 \pm 2^\circ\text{C}$ for 48 h, respectively. Following incubation, a 10 μl loopful, from each broth, was streaked onto XLD Agar supplemented with rifampicin (XLD; 80 $\mu\text{g}/\text{ml}$; Difco, Becton, Dickinson, Sparks, MD). XLDR was incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Following incubation typical colonies were present. When enrichments were positive, populations were recorded as 0.3 log CFU/ml (limit of detection).

For STEC enrichment, equal volume of (10 mL) of double strength modified buffered peptone water with pyruvate was added to the sample and incubated at $35 \pm 2^\circ\text{C}$ for 5 h. Following 5 h incubation, stock solution of rifampicin was added to standard concentration (32 μL of rifampicin to 20 mL; 80 $\mu\text{g}/\text{mL}$; Difco, Becton Dickinson, Sparks, MD) and incubated again at $42 \pm 2^\circ\text{C}$ for 18-24 h. After overnight incubation, the enrichment was streaked onto SMACR and incubated for 24 h at $35 \pm 2^\circ\text{C}$. After 24 h, if typical colonies are present, enrichment was recorded as positive, and populations were recorded as 0.3 log CFU/g (limit of detection).

4.3.6 Typing Banked Isolates and Pulse-Field Gel Electrophoresis (PFGE)

Six random isolates of *Salmonella* were recovered and saved at all sampling times by picking from plate or enrichment colonies. Colonies were selected on Day 0, 7, 28, 84, 168, 256, and 364. Pulse-Field Gel Electrophoresis (PFGE) X-bal enzyme procedure was followed according to CDC for serovar analysis. *Salmonella* Branderup H9812 was used as the reference for gel analysis.

4.3.7 Statistical Analysis

The means of the data for *E. coli* and *Salmonella* strains were calculated for the fecal samples. The statistical difference between the populations of the bacterial cocktails in the different manure type and for each time interval was evaluated using ANOVA. Multifactor analysis of variance tests for animal and pathogen interactions within each animal and each time interval was calculated. All data was processed using Microsoft Excel, 2013 and JMP 11 software (SAS® Institute Inc., Cary, NC, USA 2013). The alpha value was set at 0.05.

To assess estimated reduction rates of *E. coli* and *Salmonella* within the feces, survival data for all animals were used. To determine the population of *Salmonella* in cattle and wild animal feces, linear regression line equations for all type of tested microorganisms were calculated.

4.4 Results and Discussion

A diverse subset of five types of animal feces were used in this study. Measured sampled characteristics for each type was determined. Fecal pH values and percentage of dry matter for each sample was determined as follows: cattle, 7.07 and 15.97%; pig, 6.29 and 29.37%; waterfowl, 7.38 and 21.19%; deer, 8.03 and 43.26%; raccoon, 6.37 and 35.27%.

A cocktail of five *Salmonella* serotypes performed differently in feces from diverse animal species. Although, *Salmonella* populations in both cattle and deer feces

were noticeably similar throughout the study. At day 0, initial *Salmonella* population ranged from 5.2 to 6.1 log CFU/g in all fecal samples, and increased until day 7 in all types of fecal material with the exception of pig and raccoon. Population decline started in pig feces after day 1 and *Salmonella* could not be detected at day 14. *Salmonella* presence was found at 6.4 log CFU/g in raccoon feces on day 28 then was not detected for the remainder of the trial (day 56 to 364). At day 7, *Salmonella* population increased in three types of animal feces. *Salmonella* levels found in cattle, waterfowl, and deer feces were 1.6 log CFU/g, 1.3 log CFU/g, and 0.7 log CFU/g higher than their inoculum, respectively. At day 14, *Salmonella* levels (6.8 log CFU/g) began to decrease in waterfowl feces until day 140, with concentrations shown at 2.5 log CFU/g. *Salmonella* was undetectable in all waterfowl samples for the remainder of this study.

At day 14 and 28, *Salmonella* levels began to decrease in cattle feces, ranging from 7.0 to 7.3 log CFU/g. From 56 and 112, *Salmonella* levels declined in cattle feces from 6.5 to 5.8 log CFU/g. After four months of sampling, *Salmonella* population in cattle feces remained slightly 0.6 log CFU/g higher than starting concentrations. At day 140 and 168, *Salmonella* populations showed a decline (1.0 log CFU/g), to 4.2 log CFU/g in cattle feces. From day 196 to 308, *Salmonella* ranged from 3.0 log CFU/g to 5.0 log CFU/g. At day 308, *Salmonella* population decline was 2.2 log CFU/g lower than initially found in cattle feces, respectively. *Salmonella* was undetected on day 336 samples but from at 6.0 log CFU/g one year following the inoculation of *Salmonella* on cattle feces.

From days 14 to 56, in deer feces *Salmonella* populations increased, ranging from 6.5 to 6.9 log CFU/g. At day 84, the concentration of *Salmonella* was 1.2 log CFU/g lower than initially found on deer feces. Between day 112 and 280, deer feces samples contained *Salmonella* populations ranging from 3.9 to 5.4 log CFU/g. *Salmonella* was undetected on day 308 and 336 samples. But similar to day 364 cattle feces, *Salmonella* was enumerated at 6.5 log CFU/g one year following the inoculation of *Salmonella* on deer feces. At final sampling point day (364), *Salmonella* levels could only be enumerated in cattle and deer feces. This corresponding trend was noticed throughout the study, with *Salmonella* populations in both cattle and deer feces. *Salmonella* was able to be enumerated from cattle feces in all but one sample (18/19). While 89% (17/19) of deer samples, 26% (5/19) of pig, 32% (6/19) of raccoon, and 42% (8/19) of waterfowl samples were found to contain detectable levels of *Salmonella*. Rodriguez et al. (2006) sampled different types of farms to assess the prevalence and diversity of *Salmonella* from cattle, dairy, poultry and swine production facilities. Their findings reported 4.7% of all samples positive for *Salmonella* serovars, with swine farms (57.3%) having the highest occurrence followed by a significantly lower rates on dairy farms (17.9%), poultry farms (16.2%), and beef cattle farms (8.5%). In addition, this study investigated the diversity among serovars. *Salmonella* Anatum (48.4%) was most commonly isolated followed by *Salmonella* serovars Arizonae (12.1%) and Javiana (8.8%).

For each animal, reduction rates for *Salmonella* population initial linear decline were calculated. *Salmonella* reduction rates were determined by the number of days

for populations to achieve 1 log reduction. The resulting R^2 values for *Salmonella* ranged from 0.50 to 0.91 for all types of feces. All reduction rates and number of days needed for linear decline data are shown with equations in Table X. In cattle and deer feces, high rates of reduction were calculated. Reduction rates for *Salmonella* were 74.1 and 66.2 days, respectively. Reduction rates for pig, waterfowl, and raccoon remained below 8 days. *Salmonella* had reduction rates of 2.0, 2.2, and 7.9 days, respectively.

Previous studies of similar design have mainly used feces from cattle contaminated with *E. coli*, compared with our project survival in these studies was much shorter and detectable *E. coli* was found in cattle feces and manure-amended soils for 2 to 6 months, respectively (Jiang *et al.* 2002; Himathongkham *et al.* 1999; Wang *et al.* 1996) The objective of this project was to assess differences in persistence of STEC strains within fecal samples collected from 7 different birds and animals located within a 15 mile radius of the University of Delaware campus (Newark, DE). STEC behaved differently in feces from different animal species. At Day 0, initial STEC population ranged between 5.0 and 5.7 in all samples, and increased in all type of feces until Day 5 with the exception of raccoon and waterfowl (Figure 5-2, Table 5-2). Direct decline started in raccoon feces after inoculation and STEC could not be detected at Day 28 upon enrichment. Population increase at Day 5 reached around 0.9 log CFU/g in the cattle feces, 2.4 log CFU/g for the pig feces, and 1.7 log CFU/g in the deer feces. STEC population decline was 2.3 log CFU/g and 2.4 log CFU/g lower than initial concentration in the cattle and the deer feces at Day 364,

respectively. STEC was detected in waterfowl and pig feces until Day 224 and 196; however, the population ranged from 0 to 2.0 log CFU/g after Day 14. STEC populations could only be enumerated from the cattle and the deer feces at the end of the study on Day 364. STEC behaved statistically the same as in pig, waterfowl, and raccoon samples starting at Day 84 ($P>0.05$). In the pig samples, the population of STEC decreased up to five logs, and enumeration of STEC strains was not possible by plate counting on Day 168. Similar trends were observed in cattle and deer samples until the last sampling day. STEC survived for extended period of a time in all types of feces in this study. STEC strains survived better in the cattle and deer feces than other animal feces. Initial concentrations of pathogens ranged from 5.3 to 5.4 Log CFU/g for deer and cattle samples. No reduction below initial concentration was observed for both STEC in the deer samples until Day 84, and Day 112 in the cattle samples. At the end of the sampling period (Day 364), all samples were below the detection limit.

A total of 198 banked *Salmonella* colonies were serotyped. For identification, patterns corresponding with banked isolates were compared to patterns of the six serotypes used in the inoculum cocktail. All inoculated *Salmonella* serovars used in this study were found in at least one banked isolates sourced from fecal matter. The percentage of *Salmonella* survival varied between all six serovars, the predominant serovars found across all feces types are ordered as follows: Branderup, 44% (87/198); Anatum, 17% (34/198); Javiana, 15% (29/198); Typhimurium, 15% (29/198); Montevideo, 7% (14/198) and Newport, 2.5% (5/198).

For STEC, A total of 244 banked *E. coli* colonies were O-typed. For identification, antisera corresponding with banked isolates were compared to patterns of the 5 STECs used in the inoculum cocktail. All inoculated *E. coli* serovars used in this study were found in at least one banked isolates sourced from fecal matter expect for. The percentage of STEC survival varied between all five fecal types, the predominant o-types found across all feces types are ordered as follows: O104, 68% (167/244); O111, 28% (67/244); O145, 2% (5/244); O157, 2% (5/244); and O103, 0.0% (0/244).

In depth characterization, using molecular tools from food contamination events involving *Salmonella* and *E. coli* and environmental surveys mentioned above have provided scientists with a database of pathogenic strains. Monitoring *Salmonella* serovar presence and prevalence in wildlife is done for the overall benefit of *Salmonella* epidemiology (You *et al.* 2006). The characteristics that allow one serovar to survive over others is not well understood. Shi *et al.* (2007) reported colonization and survival of *Salmonella* on tomatoes was serovar independent, but that growth on tomatoes was serovar dependent. Serovars commonly isolated from poultry such as Enteritidis, Typhimurium, and Dublin showed slower growth on tomatoes relative to Montevideo, or Newport (Shi *et al.* 2007). This may in part explain why certain serovars continue to be associated with produce outbreaks. For this study, pathogenic serovars that were associated with vegetable outbreaks with known environmental stability were selected.

In this study, *Salmonella* and *E. coli* persisted for a minimum of 7 days in all types of feces. However, *E. coli* and *Salmonella* survived longer and were found at higher concentrations in the cattle and deer feces, when compared with the other animal feces. However, *Salmonella* was enumerated from both fecal types until the final sampling (day 364). In pig, waterfowl, and raccoon samples, the populations of *E. coli* and *Salmonella* remained low or was undetectable by enrichment. *Salmonella* was not found on pig and waterfowl beyond day 7, while, raccoon detected until day 140. In these fecal types, there was a 5 log CFU/g reduction experienced over extended period of time, ranging from days to months, which supports why agricultural and farm animals must be controlled in produce field and be supported by local and nationwide growing standards such as: no harvest zone or waiting times between harvests.

Table 5. The average *Salmonella* population and standard deviation at each sampling point for five fecal types sourced in the Mid-Atlantic United States.

Table 5 <i>Salmonella</i> populations on each animal feces in Delaware					
	Mean± SD <i>Salmonella</i> population (log CFU/g) (DE)				
Day	Cattle	Pig	Waterfowl	Deer	Raccoon
0	5.2±1.1a	5.9±0.2a	6.1±0.1a	6.0±0.1a	6.0±0.2a
1	6.4±0.8a	7.2±0.4a	7.4±0.6a	6.2±0.8a	6.9±0.5a
3	6.5±0.2a	4.8±1.2b	7.0±0.3a	6.9±0.6a	6.3±0.6ab
5	6.6±0.2a	3.7±0.8b	6.9±0.2a	6.9±0.5a	6.1±0.0a
7	7.8±0.7a	2.1±0.2c	7.4±0.3ab	6.7±1.5ab	5.7±0.6b
14	7.0±0.3a	0.0±0.0b	6.8±0.2a	6.5±0.8a	0.0±0.0b
28	7.3±0.3a	0.0±0.0c	4.0±0.3b	6.9±1.3a	6.4±0.3a
56	6.5±0.8a	0.0±0.0b	0.0±0.0b	6.7±0.6a	0.0±0.0b
84	5.8±0.5a	0.0±0.0b	0.0±0.0b	4.8±1.0a	0.0±0.0b
112	5.8±1.2a	0.0±0.0b	0.0±0.0b	4.1±1.3a	0.0±0.0b
140	4.2±0.9a	0.0±0.0b	2.5±0.9b	4.2±1.4a	0.0±0.0b
168	4.2±0.9a	0.0±0.0b	0.0±0.0b	3.9±1.3a	0.0±0.0b
196	3.8±2.3a	0.0±0.0b	0.0±0.0b	5.0±0.7a	0.0±0.0b
224	4.7±0.7a	0.0±0.0b	0.0±0.0b	3.7±1.7a	0.0±0.0b
252	5.0±0.6a	0.0±0.0b	0.0±0.0b	4.2±0.1a	0.0±0.0b
280	4.2±1.3a	0.0±0.0b	0.0±0.0b	5.4±0.3a	0.0±0.0b
308	3.0±0.6a	0.0±0.0b	0.0±0.0	0.0±0.0b	0.0±0.0b
336	0.0±0.0a	0.0±0.0a	0.0±0.0	0.0±0.0a	0.0±0.0a
364	6.0±0.1a	0.0±0.0b	0.0±0.0b	6.5±0.1a	0.0±0.0b

Means with same letter in each row are not significantly different (P>0.05)

Table 6. The average STEC population and standard deviation at each sampling point for five fecal types sourced in the Mid-Atlantic United States.

Table 6 STEC populations on each animal feces in Delaware					
	Mean± SD E. coli population (log CFU/g) (DE)				
Day	Cattle	Pig	Waterfowl	Deer	Raccoon
0	5.4±0.3a	5.8±0.2a	5.6±0.7a	5.3±0.7a	5.9±0.3a
1	6.7±0.3a	6.7±0.2a	7.4±0.1a	7.2±0.5a	5.1±0.3a
3	6.7±0.6a	4.3±0.2b	7.0±0.4a	7.1±0.4a	5.9±1.5ab
5	7.1±0.5a	3.4±2.4	6.9±0.5a	7.1±0.9a	6.1±1.3a
7	6.8±0.6a	3.4±1.7c	7.4±0.3a	7.1±1.1a	5.1±0.3b
14	7.5±0.3aa	0.0±0.0b	7.1±0.9a	7.0±0.5a	0.0±0.0b
28	6.8±0.4aa	4.8±0.2ab	5.5±0.3ab	6.8±0.4a	0.0±0.0c
56	6.6±0.2a	0.0±0.0b	5.2±0.2a	6.8±0.2a	0.0±0.0b
84	5.7±0.8a	0.0±0.0b	0.0±0.0b	3.5±0.4a	0.0±0.0b
112	6.6±0.3a	0.0±0.0b	0.0±0.0b	4.6±0.6a	0.0±0.0b
140	4.2±1.0a	0.0±0.0b	0.0±0.0b	3.9±0.3a	0.0±0.0b
168	3.9±0.5a	0.0±0.0b	0.0±0.0b	3.5±1.4a	0.0±0.0b
196	5.2±0.2a	0.0±0.0c	0.0±0.0c	2.4±1.8b	0.0±0.0b
224	4.6±0.7a	0.0±0.0c	0.0±0.0c	1.2±0.0b	0.0±0.0c
252	4.0±0.1a	0.0±0.0b	0.0±0.0b	4.5±0.1a	0.0±0.0b
280	3.3±0.8a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b
308	2.2±0.1a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b
336	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
364	6.5±2.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b

Means with same letter in each row are not significantly different (P>0.05)

Table 7: The rate of reduction and R² values for *E. coli* and *Salmonella* on five fecal types sourced in the Mid-Atlantic United States.

Table 7 Rate of reduction for <i>Salmonella</i> on each animal feces for initial linear decline				
	Day used in equation	Equation	R ²	Reduction rate for 1 log (Days)
DE/Cattle	336	$y=-0.0135x + 6.8781$	0.7364	74.1
DE/Pig	14	$y=-0.4915x + 6.4109$	0.9096	2.0
DE/WFOWL	56	$y=-0.1271x + 7.5064$	0.9107	7.9
DE/Deer	336	$y=-0.0151x + 6.7598$	0.7082	66.2
DE/Raccoon	14	$y=-0.4531x + 6.4306$	0.5057	2.2

Table 3 Rate of reduction for <i>E. coli</i> each animal feces for initial linear decline				
	Day used in equation	Equation	R ²	Reduction rate for 1 log (Days)
DE/Cattle	336	$y=-0.0156x + 6.9207$	0.7364	64.1
DE/Pig	14	$y=-0.0134x + 3.4109$	0.4536	74.6
DE/WFOWL	84	$y=-0.0237x + 5.8863$	0.6953	42.2
DE/Deer	336	$y=-0.0190x + 6.6445$	0.7510	52.6
DE/Raccoon	14	$y=-0.0137x + 3.2781$	0.4040	72.9

Manure Type	pH	Aw	Source	Appearance
Porcine	6.29	0.989	University of Delaware CANR Farm	
Bovine	7.07	0.993	Elkton, MD Farm (5 Mi. from UD)	
Raccoon	6.37	0.980	Riparian Woods Chadds Ford, PA	
Sheep	8.98	0.972	University of Delaware CANR Farm	
Goose	7.38	0.989	University of Delaware CANR Farm	
Deer	8.03	0.993	Riparian Woods Chadds Ford, PA (15 mi. from UD)	

Figure 14. Appearance and characteristics for fecal types sourced in the Mid-Atlantic United States.

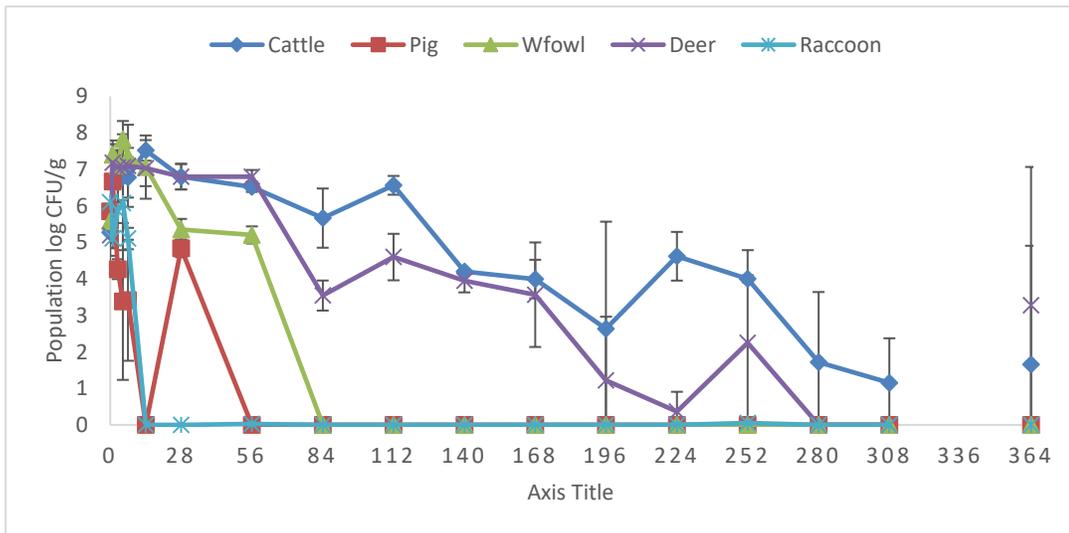


Figure 15. Populations of STEC on feces over one year period.

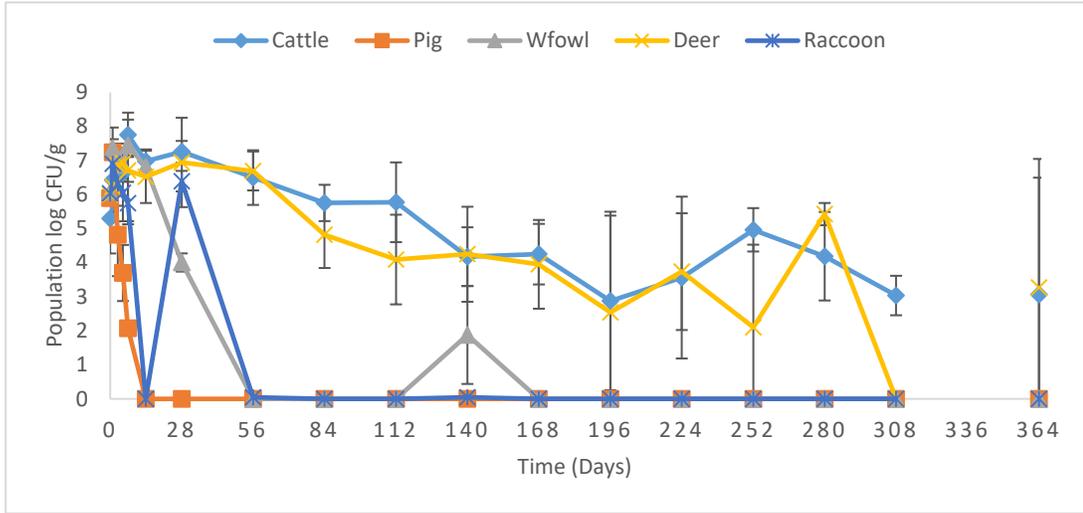


Figure 16. Populations of *Salmonella* on feces over one year period.

REFERENCES

1. Assadian NW, Fenn LB, Flores-Ortiz MA, et al. Spatial variability of solutes in a pecan orchard surface-irrigated with untreated effluents in the upper Rio Grande River basin. *Agric Water Manag* 1999;42:143-156.
2. Barton Behravesh C, Mody RK, Jungk J, Gaul L, Redd JT, Chen S, Cosgrove S, Hedican E, Sweat D, Chávez-Hauser L, Snow SL, Hanson H, Nguyen TA, Sodha SV, Boore AL, Russo E, Mikoleit M, Theobald L, Gerner-Smidt P, Hoekstra RM, Angulo FJ, Swerdlow DL, Tauxe RV, Griffin PM, Williams IT; Salmonella Saintpaul Outbreak Investigation Team. 2011. 2008 outbreak of Salmonella Saintpaul infections associated with raw produce. *N Engl J Med*. 2011 Mar 10;364(10):918-27.
3. Benjamin, L., E.R. Atwill, M. Jay-Russell, M. Cooley, D. Carychao, L. Gorski, R.E. Mandrell. 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. *Int J Food Microbiol*.165(1):65-76.
4. Bennett SD, Littrell KW, Hill TA, Mahovic M, Behravesh CB.2014 Multistate foodborne disease outbreaks associated with raw tomatoes, United States, 1990-2010: a recurring public health problem. *Epidemiol Infect*.Aug 28:1-8.
5. Brackett R. E., 1999. Incidence, contributing factors, and control of bacterial pathogens in produce. *Postharvest Biol. Technol*. 15:305-311

6. CalFERT (California Food Emergency Response Team). 2008. Investigation of the Taco John's Escherichia coli O157:H7 outbreak associated with iceberg lettuce
7. Centers for Disease Control and Prevention (CDC). 2011. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food-- foodborne diseases active surveillance network, 10 U.S. sites, 1996-2010. *MMWR Morb Mortal Wkly Rep.* 60(22):749-55.
8. Centers for Disease Control and Prevention (CDC). 2014. Trends in Foodborne Illness in the United States, 2006–2013. Accessed on April 27, 2015. <http://www.cdc.gov/foodnet/data/trends/trends-2013.html>
9. Cieslack, P.R., T.J. Barrett, P.M.Griffin, K.F. Gensheimer, G. Beckett, J. Buffington and M.G. Smith.1993. Escherichia coli O157:H7 infection from a manured garden. *Lancet.* 342(8867):367
10. Compton, J. A., J. A. Baney, S. C. Donaldson, B. A. Houser, G. J. San Julian, R. H. Yahner, W. Chmielecki, S. Reynolds, and B. M. Jayarao. 2008. Salmonella Infections in the Common Raccoon (*Procyon lotor*) in Western Pennsylvania. *J Clin Microbiol.* 46(9): 3084–3086.
11. U.S. Food and Drug Administration. 2008. Sprouters Northwest, Inc. recalls alfalfa sprout products because of possible health concern. Available at <http://www.fda.gov/oc/po/firmrecalls/sprouters0908.html>. Rockville, MD..
12. U.S Food and Drug Administration. Standards for the growing, harvesting, packing, and holding of produce for human consumption. (2015) Available at:

<https://www.federalregister.gov/articles/2015/11/27/2015-28159/standards-for-the-growing-harvesting-packing-and-holding-of-produce-for-human-consumption>. Accessed 14 March 2016.

13. Ferens, W.A. and C.J. Hovde. 2011. Escherichia coli O157:H7: animal reservoir and sources of human infection. *Foodborne Pathog Dis.* 2011. 8(4):465-87
14. Garcia SS, Ake C, Clement B, et al. Initial results of environmental monitoring in the Texas Rio Grande Valley. *Environ Int* 2001;26:465-474.
15. Gorski, L., C. T. Parker, A. Liang, M. B. Cooley, M. T. Jay-Russell, A. G. Gordus, E. R. Atwill and Robert E. Mandrell. 2013. Prevalence, Distribution, and Diversity of Salmonella enterica in a Major Produce Region of California *Appl. Environ. Microbiol.* vol. 77 no. 8 2734-2748
16. Gorski, L., C.T Parker, A. Liang, M.B. Cooley, M.T. Jay-Russell, et al. (2011) Prevalence, distribution, and diversity of Salmonella enterica in a major produce region of California. *Applied and Environmental Microbiology* 77: 2734–2748.
17. Greig, J., A. Rajić, I. Young, M. Mascarenhas, L. Waddell, and J. LeJeune. 2014. A Scoping Review of the Role of Wildlife in the Transmission of Bacterial Pathogens and Antimicrobial Resistance to the Food Chain. *Zoonoses Public Health.* 2014 Aug 30.
18. Guan, T.Y. and R. A. Holley. 2003. Pathogen survival in swine manure environments and transmission of human enteric illness--a review. *J Environ Qual.* 32(2):383-92. Review.

19. Haley et al 2009 Haley, N. J., Seelig, D. M., Zabel, M. D., Telling, G. C. & Hoover, E. A. 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS ONE
20. Hanning IB, Nutt JD, Ricke SC.2009 Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. Foodborne Pathog Dis. 6(6):635-48.
21. Harris LJ1, Berry ED, Blessington T, Erickson M, Jay-Russell M, Jiang X, Killinger K, Michel FC, Millner P, Schneider K, Sharma M, Suslow TV, Wang L, Worobo RW. 2013. A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the application of untreated soil amendments of animal origin on land used to grow produce that may be consumed raw. J Food Prot. 76(6):1062-84.
22. Harris, L.J, J.N Farber, L. R. Beuchat et al. 2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. Comp Rev Food Sci Food Safety;2:78–141
23. Himathongkham, S., S. Bahari, H. Riemann and D. Cliver. 1999. Survival of Escherichia coli O157:H7 and Salmonella typhimurium in cow manure and cow manure slurry. FEMS Microbiol Lett. 178(2):251-7.
24. Hoar, B.R., E. R Atwill, L. Carlton, J. Celis, J. Carabez and T. Nguyen. 2013. Buffers between grazing sheep and leafy cops augment food safety. California Agriculture. Apr-Jun;67(2):104-109

25. Hutchison, M. L., L. D. Walters, T. Moore, D. John, I. Thomas, and S. M. Avery. 2005. Fate of Pathogens Present in Livestock Wastes Spread onto Fescue Plots. *Appl Environ Microbiol.* Feb; 71(2): 691–696.
26. Jiang, X., J. Morgan J and M.P. Doyle. 2002. Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Appl Environ Microbiol.* 68(5):2605-9.
27. Wang, G., T. Zhao and M.P. Doyle 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl Environ Microbiol.* 62(7):2567-70.
28. Hutchison, M. L., F. A. Nicholson, K. Smith, W. C. Keevil, and T. Moore. 2000. A study of on-farm manure applications to agricultural land and an assessment of the risks of pathogen transfer into the food chain. MAFF report FS2526. Ministry of Agriculture Fisheries and Foods, London, United Kingdom.
29. Jay, M.T and G. W. Wiscomb. 2008. Food Safety Risks and Mitigation Strategies for Feral Swine (*Sus scrofa*) near Agriculture Fields, Proc. 23rd Vertebr. Pest Conf.(R. M. Timm and M. B. Madon, Eds.) Published at Univ. of Calif., Davis. 2008. Pp. 21-25.
30. Jay, M.T and G. W. Wiscomb. 2008. Food Safety Risks and Mitigation Strategies for Feral Swine (*Sus scrofa*) near Agriculture Fields, Proc. 23rd Vertebr. Pest Conf.(R. M. Timm and M. B. Madon, Eds.) Published at Univ. of Calif., Davis. 2008. Pp. 21-25.
31. Jay-Russell, M.T., A.F. Hake, Y. Bengson, A. Thiptara, and T. Nguyen. 2014. Prevalence and Characterization of *Escherichia coli* and *Salmonella* Strains

Isolated from Stray Dog and Coyote Feces in a Major Leafy Greens Production Region at the United States-Mexico Border PLoS One. 9(11): e113433.

32. Jones, D. L. 1999. Potential health risks associated with the persistence of *Escherichia coli* O157 in agricultural environments. *Soil Use Manag.* 15:76-83
33. Jones, K.E., N.G. Patel, M.A. Levy, A. Storeygard, D. Balk, J.L. Gittleman, P. Daszak. 2008. Global trends in emerging infectious diseases. *Nature*;451(7181):990
34. Keener KM, Bashor MP, Curtis PA, et al. Comprehensive review of *Campylobacter* and poultry processing. *Comp Rev Food Sci Food Saf* 2004;3:105-116.
35. Laidler, M.R., M. Tourdjman, G.L. Buser, T. Hostetler, K.K. Repp, R. Leman, M. Samadpour and W.E. Keene *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. 2013 *Clin Infect Dis.* 57(8):1129-34.
36. Luechtefeld NAW, Blaser MJ, Reller LB, et al. Isolation of *Campylobacter fetus* subsp. *jejuni* from migratory waterfowl. *J Clin Microbiol* 1980;12:4061108.
37. Mahon B, Ponka A, Hall W, et al. An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seeds. *J Infect Dis* 1997;175:876-882.
38. Meays, C.L., K. Broersma, R. Nordin, A. Mazumder and M. Samadpour. 2006. Spatial and annual variability in concentrations and sources of *Escherichia coli* in multiple watersheds. *Environ Sci Technol.* 40(17):5289-5296.

39. Patchanee, P., B. Molla, N. White, D. E Line, W. A Gebreyes. 2010 Tracking salmonella contamination in various watersheds and phenotypic and genotypic diversity. *Foodborne Pathog Dis*;7(9):1113-20
40. Pell, A.N. 1997 Manure and microbes: public and animal health problem?. *J Dairy Sci*;80(10):2673-81. Review
41. Ravva, S.V. and C.Z. Sarreal. 2014. Survival of *Salmonella enterica* in Aerated and Nonaerated Wastewaters from Dairy Lagoons. *Int J Environ Res Public Health*;11(11):11249-60
42. Rodriguez, A., P. Pangloli, H.A. Richards, J.R> Mount JR, Draughon FA. 2006. Prevalence of *Salmonella* in diverse environmental farm samples. *J Food Prot.*;69(11):2576-80.
43. Saleha AA. Epidemiological study of the colonization of chickens with *Campylobacter* in broiler farms in Malaysia: possible risk and management factors. *Int J Poult Sci* 2004;3:129-134.
44. Santo Domingo, J.W., S. Harmon, J. Bennett. 2000 Survival of *Salmonella* species in river water. *Curr Microbiol*;40(6):409-17.
45. Scallan E, R.M. Hoekstra, F. J. Angulo, R.V. Tauxe, M.A Widdowson, S.L. Roy, J.L. Jones, P.M. Griffin. 2011 Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis*. Jan;17(1):7-15
46. Shi X, Namvar A, Kostrzynska M, et al. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *J Food Prot* 2007;70:2725-2731.

47. Strauch, D., and G. Ballarini. 1994. Hygienic aspects of the production and agricultural use of animal wastes. J. Vet. Med. Ser. B-Zentbl. Veterinaermed. Reihe B Infect. Dis. Vet. Public Health 41:176-228
48. Uesugi, A.R., M.D Danyluk, R. E Mandrell, L.J Harris. 2007. Isolation of Salmonella Enteritidis phage type 30 from a single almond orchard over a 5-year period. J Food Prot;70(8):1784-9.
49. Wacheck, S., M. Fredriksson-Ahomaa, M. König, A. Stolle, and R Stephan. 2010. Wild boars as an important reservoir for foodborne pathogens. Foodborne Pathog Dis.;7(3):307-12.
50. Wilkes, G., J. Brassard, T.A. Edge, V. Gannon, N. Gottschall, C. C. Jokinen , T. H. Jones, I.U Khan, R. Marti R, M.D. Sunohara, E. Topp , D.R. Lapen. 2014. Long-term monitoring of waterborne pathogens and microbial source tracking markers in paired agricultural watersheds under controlled and conventional tile drainage management. Appl Environ Microbiol;80(12):3708-20.
51. You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. Appl Environ Microbiol. 72(9):5777-83.
52. Ziemer CJ, Bonner JM, Cole D, Vinjé J, Constantini V, Goyal S, Gramer M, Mackie R, Meng XJ, Myers G, Saif LJ. 2010. Fate and transport of zoonotic, bacterial, viral, and parasitic pathogens during swine manure treatment, storage, and land application. J Anim Sci. Apr;88(13 Suppl):E84-94.

Chapter 5

OBSERVATIONS OF *E. COLI* ISOLATES IN RESPONSE TO SIMULATED GASTRIC FLUID

5.1 Abstract

Widespread fecal contamination of the environment (soil or water) by domestic and wild animals provides a continuing source of EHEC in the agricultural environment, and risk of contamination to a wide variety of raw foods. Land applied manures or biosolids are also typical sources of these fecal pathogens. Infection usually occurs when food or water is ingested that has been contaminated unknowingly with feces containing a pathogenic strain of *E. coli*. Following environmental isolates of *E. coli* suspended in manure at an average of 9.2 ± 0.3 log *E. coli* CFU/ml, each were assessed for viability in a simulated gastric fluid (SGF) assay. Subsequent exposure to spinach plants showed a rapid decline and after 24 hours at an average of 3.7 ± 1.2 log *E. coli*/ml were recovered from the leaves. The majority of *E. coli* isolates (14/17, 82%) were above the detection limit (0.8 log *E. coli* CFU/ml) initially after being exposed to SGF. After 120 minutes post-exposure to SGF, the percent reduction of *E. coli* population on plant surfaces ranged from 100 to 73.5%. These findings will aid in our understanding of *E. coli* strain survival on plant surfaces and host pathogenicity.

5.1 Introduction

Enteric pathogenic bacteria are exposed to extremely acidic conditions within the gastrointestinal tract prior to causing infection (Lin *et al.*, 1996; Tamplin *et al.*, 2005; Koo *et al.*, 2000). Increased gastric pH within humans has been directly linked

to decreased protection against infections caused by foodborne pathogens such as *Salmonella* and *Campylobacter* spp. (Holt 1985 and Schiraldi *et al.*, 1974). *E. coli* harbor genetically encoded proteins that allow them to be resistant to acidic conditions as well as lactose, which are two necessary requirements for survival inside the mammalian host (Lin *et al.*, 1996; Blatner *et al.*, 1997, Lawrence and Ochman, 1998). Two previous studies have shown the effectiveness of generic *E. coli* in predicting *Salmonella* and *E. coli* O157:H7 persistence and slurry application in laboratory settings (Park *et al.*, 2013, Natvig *et al.*, 2002, Park *et al.*, 2015).

Pre-harvest risks for contamination of produce include application of raw manure as fertilizer as well as the presence of domestic and wild animals in the field, which are sources of *E. coli*. Manure or sewage are typical sources of these fecal pathogens (Hill, 2013). Outbreaks involving fecal-contaminated water have been reported and is one of the main routes of transmission for foodborne pathogens on fresh produce (Hanning *et al.*, 2009); Harris *et al.*, 2013). Infection usually occurs when food or water is ingested that has been contaminated with feces containing a pathogenic strain of *E. coli*. Foodborne outbreaks as a result of environmental microbial contamination have growing public health implications and financial costs. The projected total economic cost due to produce-associated foodborne illnesses is estimated to be more than \$70 billion (Scharff, 2012). In two unrelated *E. coli* O157:H7 outbreaks in 2005 and 2006, lettuce irrigated with water contaminated by cattle feces sickened 135 and 71 people in Sweden and the U.S., respectively, (CalFERT, 2008; Soderstrom *et al.*, 2008). A single gene is insufficient to convert commensal to pathogenic *E. coli*; instead, a combination of genes encoding toxins, colonization factors and other functions are required to make *E. coli* pathogenic

(Kaper *et al.*, 2004). Although more than 200 serotypes of *E. coli* can produce shiga-toxin, most of these serotypes do not contain the LEE pathogenicity island and are not associated with human disease. (Hill, 2013). This has led the use of Shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC) as general terms for any *E. coli* strain that produces *Stx*. The term EHEC is used to denote only the subset of *Stx*-positive strains that also contain the LEE. (Hill, 2013).

Survival of *E. coli* in water ranges widely from a period of a few days to 12 weeks or more depending on levels of predation, temperature, light, pH, competition, and various other biotic and abiotic factors (van Elsas *et al.*, 2011). After release into the non-host environment survival can be affected by extrinsic factors such as temperatures and acid stress, damaging UV, and nutrient competition. There are also important intrinsic elements encoding for attachment and multiple stress resistance genes, which may extend survival when exposed to inhospitable environments in soil, water, and on plants. The survival capabilities of manure-borne *E. coli* is affected by temperature, moisture, pH, solar radiation, diet of animal, strain, and type of soil (Van Kessel *et al.*, 2007). This view has been challenged by a number of studies suggesting that *E. coli* can, under the right conditions, persist or even reproduce outside of the gut environment (Hill, 2013). This has been demonstrated in tropic water and soils, but also speculated or shown to occur in temperate fresh water, beach sand, soil, streambeds, and in aquatic blooms (Hill, 2013). Additionally, it has been shown that under stressful conditions, *E. coli*, including pathogenic strains, can enter a “viable but nonculturable” (VBNC) state in which the organism remains alive but is not detected by conventional culture methods (Hill, 2013). These examples of unusual persistence of *E. coli* call into question its use as an indicator organism (Hill, 2013).

As an increasing number of illnesses have been attributed to fruits and vegetables associated with the survival of pathogenic bacteria in the pre-harvest environment, microbiologists have questioned the role of the plant as a host in the survival of these organisms (van Elsas *et al.*, 2011). The pre-harvest environment is full of stressful biotic and abiotic factors that may lead to bacterial decay or may allow the organism to relish the antagonistic environment; scientists have hypothesized both situations for some time (van Elsas *et al.*, 2011). In this study spinach plants were inoculated with *E. coli* isolates and after 24 hours on the plants, the same bacteria were subjected to the SGF to address the potential role that plants may have on adaptation or fitness of bacteria in the pre-harvest environment. The totality of environmental conditions that allow a bacterium to survive on a foliar surface and then cause gastrointestinal disease is not fully understood. This work provides a snapshot into one situation comparing the potential fitness and adaptation of several *E. coli* isolates.

Evaluation of the response of bacteria to simulated human gastric fluid (SGF) is one means of comparing the relative degree of acid resistance in different bacterial strains. Here we evaluated differences in bacterial response to SGF as a function of strain origin. Strains originated from 1) bovine manure (UD dairy) that was used to irrigate lettuce, spinach, and tomatoes in field trials conducted at the College of Agriculture and Natural Resources Farm in 2012, 2013, and 2014; from 2) the leafy greens or tomato fruits collected during those trials; or from 3) laboratory cultures, where a wild-type pathogenic *E. coli* was compared to a mutant *rpoS* deficient strain. Several genes have been correlated with pathogenicity and virulence in EHEC isolates, and those genes identified in these isolates are listed in Table 2. In theory, the expected findings will help identify the prototypical *E. coli* strain that is able to

survive on plant surfaces and tolerate acidic conditions. This objective will help gather additional knowledge of the relationship between *E. coli* survival outside a host and pathogenicity. Studies assessing the dynamic environment of agriculture water described here may help understand agricultural water and the challenges that food safety experts, companies and regulators face when evaluating irrigation water for the transmission of fecal contamination

5.2 Materials and Methods

5.2.1 Preparation of Manure Water Contaminated with *E.coli*

Fresh manure samples (500g) were collected at random from the University of Delaware Dairy Farm. Manure (11g) was added to 1L of deionized water. The sample was agitated for 2 minutes, allowed to sit for approximately ten minutes at room temperature, agitated and again for 2 min. Serial dilutions of manure water were performed in Phosphate Buffer Saline (Fisher Bioreagents, Fair Lawn, NJ) for enumeration of *E.coli* on Tryptone Bile X-Glucuronide agar (TBX, Oxoid, UK) and incubated at 37°C for 24 hrs. Manure slurry was autoclaved and stored at 4°C before use

5.2.2 Preparation of Bacterial Inoculums

E. coli strains and Ampicillin or Nalidixic acid (Fisher Bioreagents, Fair Lawn, NJ) resistant EHEC, stored at -80 °C, were streaked onto tryptic soy agar or Tryptic Soy Agar with Ampicillin or Nalidixic acid (TSAA/N; 80 µg /mL; Difco, Becton, Dickinson, Sparks, MD). Agar plates were incubated at 37 ± 2 °C for 24 ± 2 h. One isolated colony of the strains was transferred into tryptic soy broth with Nalidixic acid (TSBN; Difco, Becton, Dickinson, Sparks, MD) or into manure water

and incubated at 37 ± 2 °C for 24 h. After incubation, samples were serially diluted and plated on Tryptone Bile Agar (TBX) (Oxoid, UK) and TBXN agar in duplicate and incubated at 37°C for 24hrs. A flow chart for these method can be found in Figure 1.

5.2.3 Preparation of Simulated Gastric Fluid

The simulated gastric fluid (SGF) was prepared as previously described by (Ling *et al.* 2008) with slight variation. In brief, the composition of the synthetic gastric juice included 8.3 g proteose-peptone, 3.5 g glucose, 2.05 g NaCl, 0.6 g KH₂PO₄, 0.11 g CaCl₂, 0.37 g KCl, 0.05 g bile salts, 0.1 g lysozyme, 13.3 mg pepsin, in 1 L Millipore-filtered water, pH adjusted to 1.6 with 6 N HCl. (Tamplin 2005). SGF was autoclaved and stored at 4°C before use. The lysozyme and pepsin were added fresh to sterile SGF.

5.2.4 Spinach Inoculation

Using the colony lawn inoculation protocol previous described by Theofel *et al.* (2009). The bacterial cultures were collected in 10 ml of manure water (CANR Dairy manure/Millipore-filtered deionized water, Newark, DE), by loosening the cells with a sterile loop (ThermoFisher Scientific, Mullica Hill, NJ). Loosened bacterial cells were collected with a sterile pipette. Both broth and agar preparations were washed and centrifuged (Beckman Coulter, Brea, CA) twice at 11,000 rpm (12,800 \times g) for 10 min and washed with manure water before use. The bacterial cultures were diluted in 0.1% peptone water (BD, Franklin Lakes, NJ) to the desired concentration directly before inoculation. Six-week old spinach plants var. Melody (2B Seeds,

Broomfield, CO) or SGF (n=6 of 3 pooled plants) were inoculated with 100µl (10x 10µl drops) of 8-9 log *E. coli* cfu/ml of each strain and allowed to dry for 0-24 hours. Recovered bacteria were enumerated on TBX agar and incubated at 37°C for 24 hours (Oxoid, UK).

5.2.5 Simulated Gastric Fluid Assay

E. coli isolates were recovered from manure and leafy greens in previously conducted field trials, conducted in 2013 and 2014 at the College of Agriculture and Natural Resources farm (Newark, DE). Seventeen *E. coli* isolates, including 2 laboratory-control strains, were individually compared for survival on spinach leaves (0, 24 hrs.) first and then in SGF at pH 1.6 (0, 2, and 5 hrs.) or in 0.1% peptone water. Spinach leaves were aseptically removed from plants and directly transferred into 50 ml Centrifuge tubes (Corning, NY) containing 40 ml of pre-warmed SGF at 37°C shaking at 250 rpm for 0-5 hours. Each tube was sampled immediately by removing 1 ml and adding that to 1ml of Dey-Engley neutralizing broth (BD, Franklin Lakes, NJ) and placed on ice. Samples were serially diluted in 0.1% peptone water and plating on TBX agar and incubated at 37°C for 24 hours. Samples were taken at 0, 2, and 5 hours following introduction of leaves and bacterial cultures to gastric fluid. The same protocol above was repeated for 100µl (x log) of each *E. coli* strain originally grown in 10 ml of manure slurry. Log reduction at 120 minutes and 300 minutes was calculated by using the following equation: $L = \log_{10}(A) - \log_{10}(B)$. Where A is the number of *E. coli* before treatment and B is the number of *E. coli* after treatment. Log reduction values were converted to percent reduction using the next equation: $Percent\ Reduction = (1 - 10^{-L}) \times 100$

5.2.6 PCR Analysis

E. coli samples were grown overnight in LB broth at 37°C and suspended cells from a single colony from each culture was placed in 50 µL nuclease water before being used in the following PCR assay. Manual extraction of the DNA was performed using 1:1 phenol-chloroform. DNA concentration adjusted to approximately 100ng/uL and stored at -20°C. Each isolate was tested in four multiplex PCR assays designed to screen for a total of fifteen different virulence factors (Tables 1 and 2). Two EHEC multiplex PCR assays (LeStrange *et al.* 2017) were performed for the *eaeA*, *ehxA*, *espA*, *espP*, *katP*, *stcE*, *stx1*, *stx2* genes. In brief, the 25uL reaction mixes contained 12.5uL of the Platinum® Multiplex Master Mix (Life Technologies ; catalog number 4464268), 4uL of the mixed primers (final concentrations of 50nM; 4uL total at 2.5uM each), 2.5µL DNA (final concentration of 10nM), and 6uL ultrapure water. *E. coli* O157:H7 strain 4407 possessed all eight virulence genes and was used as the positive control in the two assays. The initial activation step lasted two minutes at 96°C and was followed by 25 cycles with a 30-second denaturation at 96°C, a one minute annealing step at 60°C, and a 50-second extension step at 72°C. A final extension was set for ten minutes at 72°C.

5.2.7 Statistical Analysis

The average *E. coli* population at each time point within the SGF assay were calculated. Within each assay, the statistical difference between the populations of *E. coli* for each time interval were evaluated using ANOVA. Multifactor analysis of variance tests for SGF and strain interactions within plant and non-plant assays and each time interval was calculated. All data was processed using Microsoft Excel, 2013

and JMP 11 software (SAS® Institute Inc., Cary, NC, USA 2013). The alpha value was set at 0.05.

5.3 Results

Spinach plants were inoculated with average of 9.2 ± 0.3 log *E. coli* CFU/ml, subsequent drying for 24 hours resulted in a 3.7 ± 1.2 log *E. coli*/ml differential when recovered from the leaves. To ensure accuracy comparing survival of *E. coli* strains side by side, percent reductions of populations at various time points after SGF exposure were calculated to the 0 min value.

All strains except three (14/17 82%) were above the detection limit (0.8 log *E. coli* CFU/ml) after 0 minutes exposure to SGF was observed. However, after 300 minutes exposure to SGF (pH 1.6), 11/17 (65%) *E. coli* strains inoculated onto spinach leaves were below the detection limit (0.8 log *E. coli* CFU/ml). Many isolates were not able to survive up to 5 hours in gastric acid especially when exposed to spinach leaves. While these results are evidence bacterial reduction can occur due to gastric acid, it also is clear that extended periods of exposure to low pH environments does not completely eliminate high concentration of generic or pathogenic *E. coli*. Field and manure strains inoculated into bovine slurry showed no significant reduction following 120 minutes in SGF. Strain 52 (wild-type) was *rpoS*⁺ and strain 55 (*rpoS*-mutant) both declined significantly ($p < 0.001$) following inoculation of SGF in both plant and non-plant control. Data from the *rpoS*⁺ wild-type strain showed it was not able to provide acid resistance within in this experimental format. Manure and field isolates survived on leaves and in synthetic gastric juice better than laboratory-strains and or wild-type, suggesting these isolates may have enhanced acid resistance. At each sampling time, levels not connected by the same letter are significantly different.

Within the field *E. coli* group, *E. coli* strains 35, 205, 275 concentrations in SGF were 1-4 log higher than *E. coli* 159, 172, 355 at each time point, which were nearly undetectable after 0, 120 and 300 minutes. Likewise within the manure *E. coli* group, *E. coli* strains 24 and 320 concentrations in SGF were higher between 0-300 minutes than *E. coli* 2 and 261. Conversely, these three strains 159, 172, 355 did not survive well in SGF following plant inoculation but were able to survive SGF longer when directly exposed following incubation in bovine slurry.

Following 120 minutes of exposure to SGF, the majority of *E. coli* isolates were below the limit of detection (0.8 log *E. coli* CFU/ml). *E. coli* 234 (decreased by 73.2%) showed the best at surviving acidic conditions for the first 120 mins of exposure, while *E. coli* strains 275(81.3%), 320(85.3%), 24(87.1%), 21 (91.3%), 261 (93.3%), 22(93.6%), 35 (4.9%) and 304 (95.1%) were reduced moderately less than *E. coli* strains 2, 52, 55, 159, 172, and 355 were reduced to almost below the detection limit at this time point. Conversely, at the same time point, the resulting percent reduction of *E. coli* population suspended directly in SGF with no spinach ranged from 100 to -16.8% reduction. *E. coli* strain 205 (-16.8%) showed no decline after two hours exposed to SGF. Likewise, *E. coli* strains 35(7.84%) and 24(18.5%), showed to be better at surviving acidic conditions for the first 120 mins of exposure While, *E. coli* strains 21(51.6%), 22(53.8%), and 320(57.1%) showed almost half of the initial population of cells still remaining after 2 hours. Similarly, the *E. coli* strains 2, 52, 55, 159, 172, and 355 that did not perform well in the assay where plants were inoculated, but the bactericidal activity was less extreme in the non-plant experiments.

After 300 minutes of exposure to SGF, the percent reduction of *E. coli* population on plant surfaces due to SGF ranged from 100 to 63.1% reduction. *E. coli*

strain 275 which performed moderately at the 120 minute time point was only reduced by 63.1% after 5 hours of SGF exposure, survival unlike than all other *E. coli* strain tested in this project. Likewise, within the non-plant control group *E. coli* strain 24 was only reduced by 61.8% after 5 hours of SGF exposure and the remaining percent reduction from *E. coli* population not inoculated on plants ranged from 100 to 61.8% reduction.

Out of a total of 198 environmental isolates tested for the eight EHEC genes, 135 (68.2%) had one or more EHEC genes. Sixty-one isolates (69.3%) of these contained *stx1*, *stx2* or *eaeA*. The gene *stx1* had a prevalence rate of 6.9% (14 isolates of the 198), *stx2* at 26.1% (53), and *eaeA* at 1.5% (3). The majority of isolates had either one or two genes (30% and 10% respectively). One environmental isolate contained seven genes, and no isolate contained all eight. The multiplex findings for the *E. coli* strains used in this experiment are listed in Table 9. One commonality that was present in all of strains that survived better in SGF than others, was the presence of the *stx1* gene (Table 2). Although, EHEC 52 and 55 also had the *stx1* and *stx2* but did not perform well under the acidic conditions in this experimental format.

5.4 Discussion

Roering et al., (1999) observed a 1.7 to 2.8 log reduction in *E. coli* O157:H7 after two hours in SGF. Previous experiments involving SGF using two different recipes observed inactive rates of -0.7 to -1.3 (Koo *et al.*, 2000; Tamplin, 2005). Gordon and Small calculated that bacteria can be subjected to pH 2.5 for 2 hours due to gastric emptying and fasting. Here, bacterial isolates were exposed to a stronger gastric acid, with or without a conditioning period on a leaf. The work presented here is unique in the inclusion of *E. coli* isolates naturally present in dairy manure and the

study of these organisms from a leaf into SGF. The findings from this study show how *E. coli* persist both on edible plant surfaces before surviving extreme acid stress for extended periods. The observed survival in the non-plant control for most *E. coli* strains showed spinach did not enhance survival at pH 1.6 for 5 hours. However, a few strains did show the ability to tolerate severe acid stress and will be further investigated by this research group in depth. In addition, *E. coli* isolated from manure tended to survive better in both non-plant and plant experiments. Although, these differences were not found to be significant. The capacity to tolerate acid may permit pathogens to survive for longer in acidic foods or during food processing until bacteria are consumed (Lin *et al.*, 1996). Previous studies have focused on direct inoculation into the SGF or onto meat this project uses a vegetable as the contaminated food commodity to add to the assay. These observations add to our knowledge around acid tolerance and support the importance of the function of acid resistance in the ability to cause foodborne illness infection in the gastrointestinal tract of humans.

Simulating the relationship between acidic conditions and *E. coli* survival in the gut will build on previous knowledge available but it is important to remember this is only a small-scale model and this should be understood considering a model cannot provide 100% assurance about the persistence of *E. coli* isolates in the Gastrointestinal Tract. However, our project used controlled settings to estimate *E. coli* survival that facilitated our findings in a way that this project could differentiate between isolates used in our simulated approach.

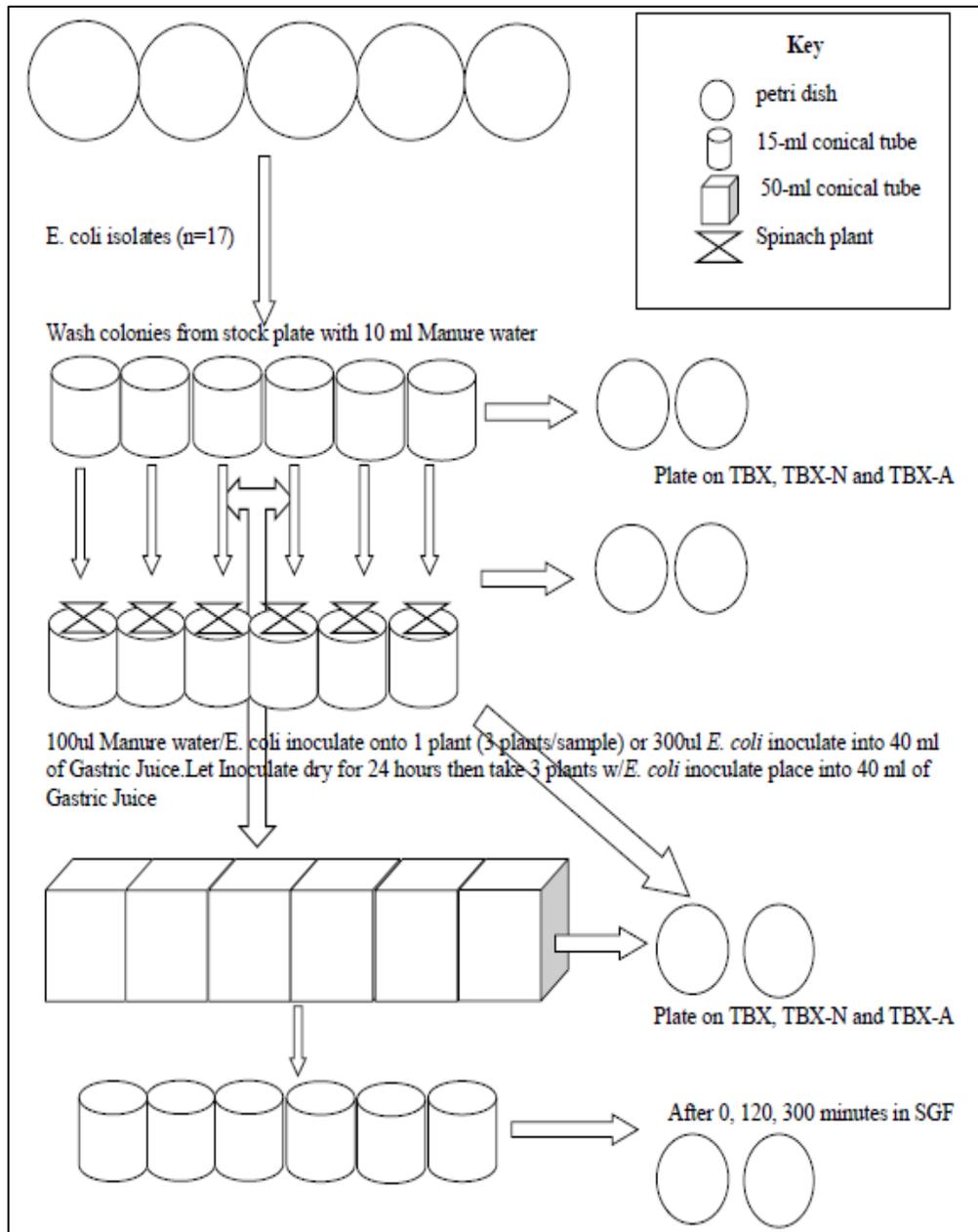


Figure 17. Flowchart depicting the procedure for culturing *E. coli* isolates and inoculation of spinach and SGF.

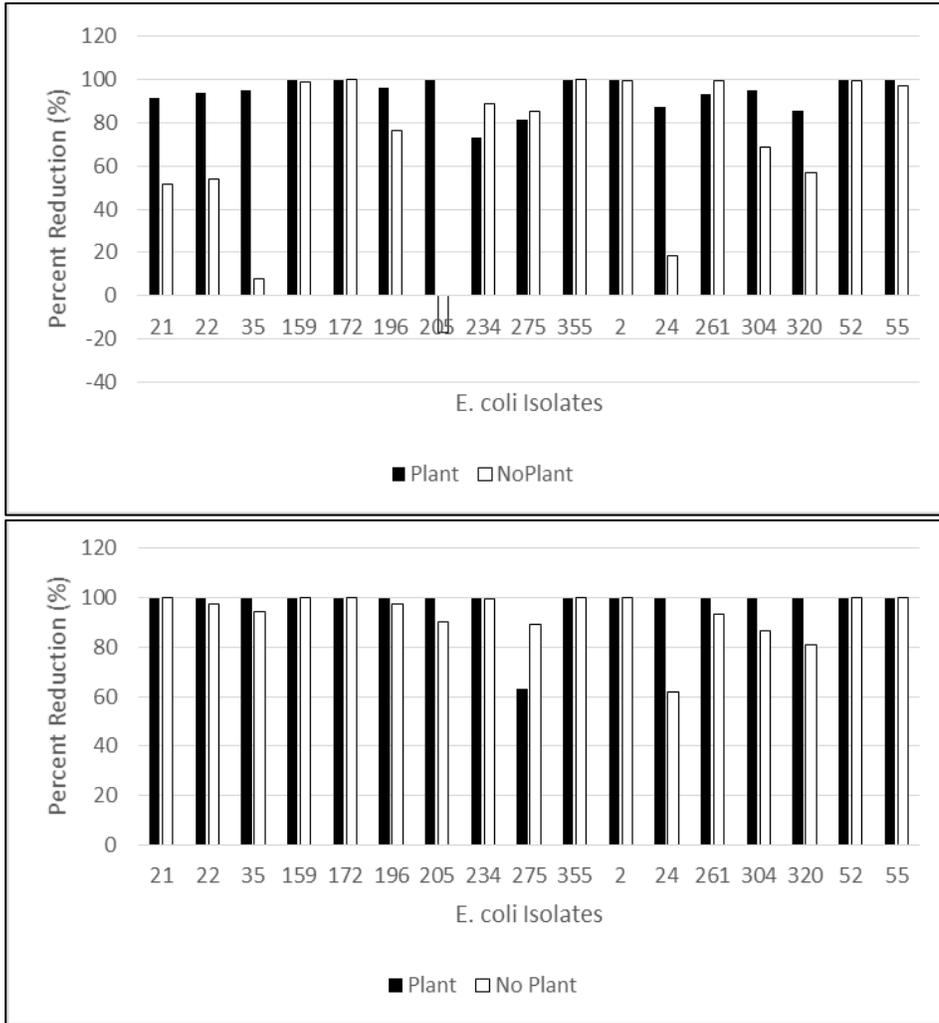


Figure 18. Calculated percent survival for *E. coli* isolates after 2 hours (top) and 5 hours (bottom) exposure to simulated gastric fluid.

Table 8. Multiplex Assays for the screening of Eight EHEC virulence genes.

Multiplex Assay Numer	Virulence Gene	Description	Primer Sequence (F)	Primer Sequence (rev)	Amplicon size	Reference
1	<i>katP</i>	catalase-peroxidase	GCG CCA GTG GTG GTC AGC AA	ATA TCG GGC TGC CCG TCC CA	914	Bustamante et al., 2011
	<i>stcE</i>	zinc metallo-protease	GGC TCC GGA GGT GGG GGA AT	GAA GCC GGT GGA GGA ACG GG	399	Bustamante et al., 2011
	<i>ehxA</i>	haemolysin	ACA GCT GCA AGT GCG GGT CTG	GGG ATG CAC TGG AGG CTG CA	262	Bustamante et al., 2011
	<i>stx1</i>		ATA AAT CGC CAT TCG TTG ACT	AGA ACG CCC ACT GAG ATC ATG	180	Paton & Paton, 1998
2	<i>espP</i>	extracellular serine protease	GCT GGC AAC CAG CAA CAG CG	CGG TAG CCC GCT TCT GCA CC	774	Bustamante et al., 2011
	<i>eaeA</i>		GAC CCG GCA CAA GCA TAA GC	CCA CCT GCA GCA ACA AGA GG	384	Paton & Paton, 1998
	<i>stx2</i>		GGC ACT GTC TGA AAC TGC TCC	TCG CCA GTT ATC TGA CAT TCT	255	Paton & Paton, 1998
	<i>espA</i>		GCC AAA CTT CCT CAA GAC GTG	CAC CAG CGC TTA AAT CAC CAC	100	Paton & Paton, 1998

Table 9. Distribution of EHEC genes, source and sample day for *E. coli* isolates.

<i>E. coli</i> isolates	EHEC genes	Source	Sample day
2	None	Manure	0
21	<i>espA</i>	Inoculated tomato	0
22	None	Inoculated tomato	0
24	<i>stx1, stx2</i>	Manure	0
35	<i>eaeA, ehxA, espA, espP, katP, stx1, stx2</i>	Inoculated lettuce	10
159	None	Inoculated spinach	10
172	<i>espA</i>	Inoculated spinach	10
196	None	Inoculated lettuce	10
205	<i>stx1</i>	Inoculated lettuce	10
234	<i>stx2</i>	Inoculated tomato	0
261	<i>ehxA, stx1</i>	Manure	0
275	<i>ehxA, stcE, stx1, stx2</i>	Inoculated tomato	7
304	<i>stx2</i>	Manure	0
320	<i>ehxA, stx1, stx2</i>	Manure	0
355	None	Tomas-Callejas et al., 2011	-
EHEC 52	<i>eaeA, ehxA, espA, espP, katP, stcE, stx1, stx2</i>	Gift from Dr. Manan Sharma	-
EHEC 55	<i>eaeA, ehxA, espA, espP, katP, stcE, stx1, stx2</i>	Gift from Dr. Manan Sharma	-

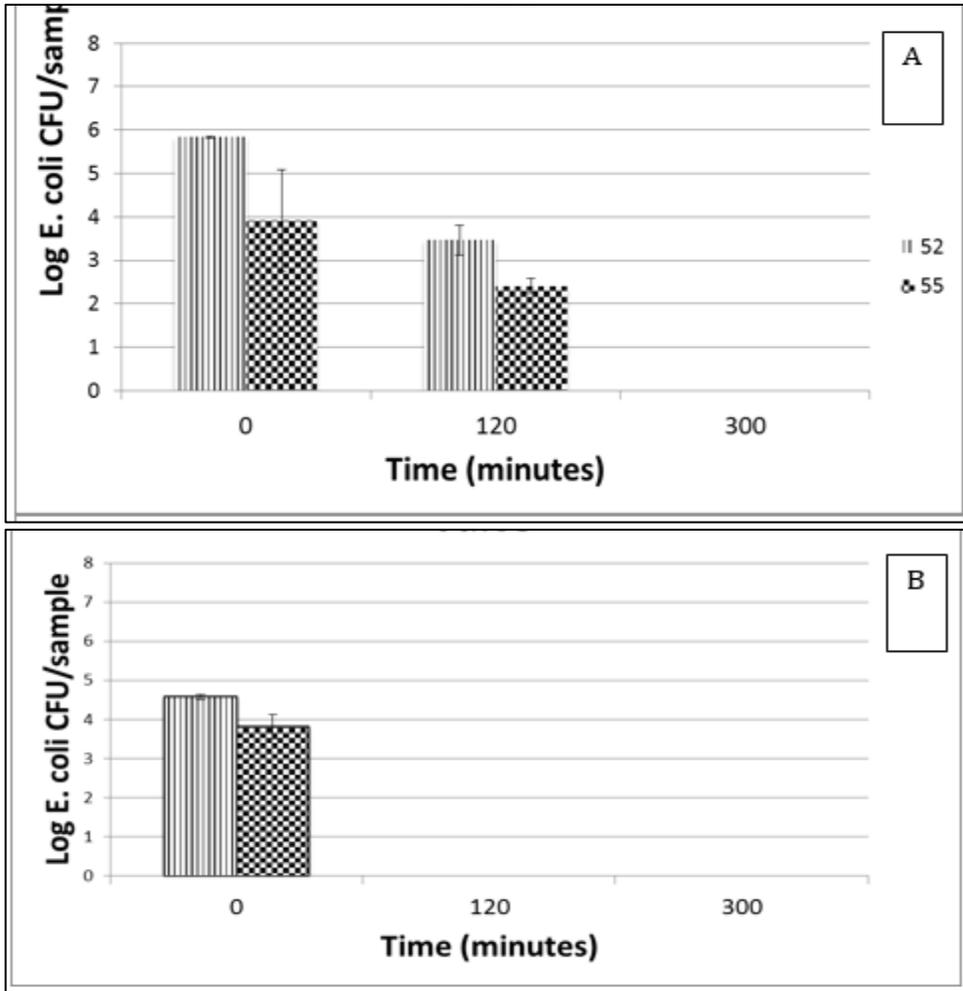


Figure 19. Persistence of laboratory and wild-type EHEC following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an *.

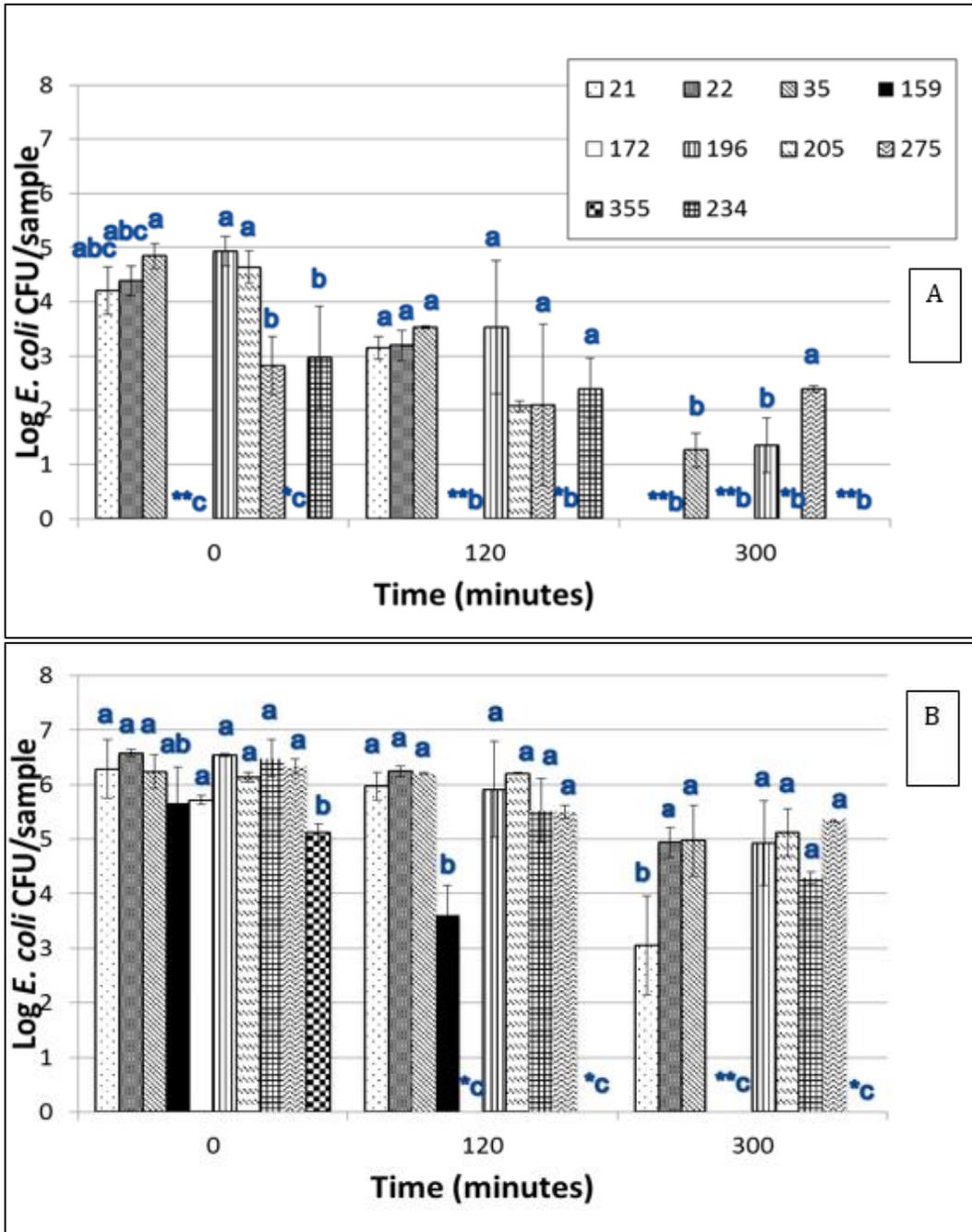


Figure 20. *E. coli* persistence following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an *.

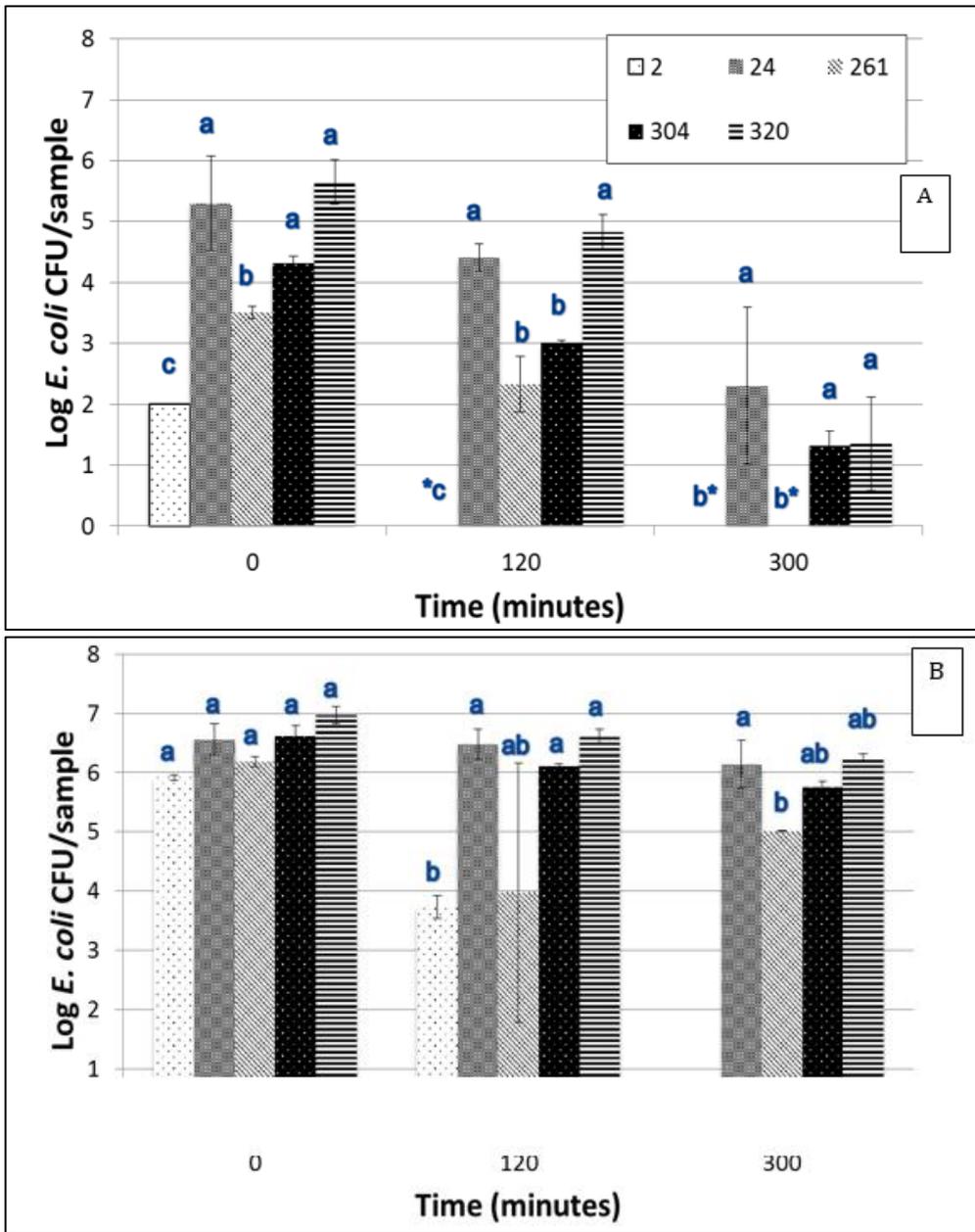


Figure 21. *E. coli* persistence following inoculation onto spinach plants prior, (A) or inoculated directly into (B) to the SGF challenge for up to 300 min. At each sampling time, levels not connected by the same letter are significantly different. Samples below the limit of detection at 0.8 log CFU/ml are indicated with an *.

REFERENCES

1. Blattner F.R., G. Plunkett III, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau and Y. Shao. 1997 Sep The complete genome sequence of *Escherichia coli* K-12. *Science*. 277(5331):1453-1462. *Bull World Health Organ*. 51(4):349-52.
2. Bustamante, A.V., A.M. Sanso, P.M. Lucchesi and A.E. Parma. 2011 Multiplex PCR assay for the detection of five putative virulence genes encoded in verotoxigenic *Escherichia coli* plasmids. *Curr. Microbiol*. 62(5):1411-1415.
3. CalFERT (California Food Emergency Response Team). 2008. Investigation of the Taco John's *Escherichia coli* O157:H7 outbreak associated with iceberg lettuce
4. Dirk van Elsas, J., A.V. Semenov, R. Costa and J.T. Trevors. 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. 5(2): 173–183. Gastric abnormalities in cholera: epidemiological and clinical considerations
5. Gordon, J., and P. L. Small. 1993. Acid resistance in enteric bacteria. *Infect. Immun*. 61:364-367.
6. Hanning IB, Nutt JD, Ricke SC. 2009 Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathog Dis*. 6(6):635-48.

7. Harris LJ1, Berry ED, Blessington T, Erickson M, Jay-Russell M, Jiang X, Killinger K, Michel FC, Millner P, Schneider K, Sharma M, Suslow TV, Wang L, Worobo RW. 2013. A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the application of untreated soil amendments of animal origin on land used to grow produce that may be consumed raw. *J Food Prot.* Jun;76(6):1062-84.
8. Hill S. 2013. Virulence factors in fecal *Escherichia coli* from humans and animals. Ontario, Canada: The University of Guelph;
9. Holt P.R., The small intestine. 1985 *Clin. Gastroenterol.*4):689-723. Review.
10. Kaper J, Nataro J, Mobley H. Pathogenic *Escherichia coli*. *Nature Reviews: Microbiology* 2004; 2: 123-40.
11. Koo, J., A. DePaola, and D. L. Marshall. 2000. Effect of simulated gastric fluid and bile on survival of *Vibrio vulnificus* and *Vibrio vulnificus* phage. *J. Food. Prot.* 63:1665-1669.
12. Lawrence J.G. and H. Ochman. 1998. Molecular archaeology of the *Escherichia coli* genome. *Proc Natl Acad. Sci. U S A.* 95(16):9413-7.
13. LeStrange, K., S. M. Markland, D.G. Hoover, M. Sharma and K.E. Kniel. 2017. An evaluation of the virulence and adherence properties of avian pathogenic *Escherichia coli*. *One Health.* 4:22-26.
14. Lin, J., M.P. Smith, K. C. Chapin, H.S. Baik, G. N. Bennett, and J. W. Foster. 1996 Mechanism of acid resistance in Enterohemorrhagic *Escherichia coli*. *Appl Environ. Microbiol.* 62(9):3094-3100.

15. Ling, J., M. Sharma and A.A. Bhagwat. 2008. Role of RNA polymerase sigma-factor (RpoS) in induction of glutamate-dependent acid-resistance of *Escherichia albertii* under anaerobic conditions. *FEMS Microbiol Lett.* 283(1):75-82
16. Natvig, E.E., S.C. Ingham, B.H. Ingham, L.R. Cooperband, T.R. Roper TR. 2002. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. *Appl Environ Microbiol* 68:2737–2744.
17. Park S., S. Navratil, A. Gregory, A. Bauer, I. Srinath, B. Szonyi, K. Nightingale, J. Anciso, M. Jun, D. Han, S. Lawhon and R. Ivanek. Multifactorial Effects of Ambient Temperature, Precipitation, Farm Management, and Environmental Factors Determine the Level of Generic *Escherichia coli* Contamination on Preharvested Spinach *Appl. Environ Microbiol.* 2015 Apr; 81(7): 2635–2650.
18. Park, S., S. Navratil, A. Gregory, A. Bauer, I. Srinath, M. Jun, B. Szonyi, K. Nightingale, J. Anciso, R. Ivanek. 2013. Generic *Escherichia coli* contamination of spinach at the preharvest level: the role of farm management and environmental factors. *Appl Environ Microbiol* 79:4347–4358.
19. Paton, A.W., P.A. Manning, M.C. Woodrow and J.C. Paton. 1998. Translocated intimin receptors (Tir) of Shiga-toxigenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from

- patients with hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. *Infect Immun.* 66(11):5580-5586.
20. Roering, A. M., J. B. Luchansky, A. M. Ihnot, S. E. Ansay, C. W. Kaspar, and S. C. Ingham. 1999. Comparative survival of *Salmonella typhimurium* DT104, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in preservative-free apple cider and simulated gastric fluid. *Int. J. Food Microbiol.* 46:263-269.
21. Schiraldi O., V. Benvestito, C. Di Bari, R. Moschetta and G. Pastore. 1974
22. Söderström, A., P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. Blide Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G. Allestam, I Hedenström, L. Ledet Muller, and Y. Andersson. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog Dis.* 5(3):339-349.
23. Tamplin, M. L. 2005 Inactivation of *Escherichia coli* O157:H7 in simulated human gastric fluid. *Appl. Environ. Microbiol.* 71(1):320-325.
24. Theofel, C.G. and L.J. Harris. 2009. Impact of preinoculation culture conditions on the behavior of *Escherichia coli* O157:H7 inoculated onto romaine lettuce (*Lactuca sativa*) plants and cut leaf surfaces. *J Food Prot.* 72(7):1553-9.
25. Van Kessel, J.S., Y.A. Pachepsky, D.R. Shelton and J.S. Karns. 2007 Survival of *Escherichia coli* in cowpats in pasture and in laboratory conditions. *J Appl Microbiol.* 103(4):1122-7.