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Salmonella Survival and Transport in the Environment:

Implications for Produce Safety and Human Health Risk

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By

Debbie Lee

B.A. Stanford University, 2011

Doctor of Philosophy

Environmental Health Sciences

Advisor: Karen Levy, PhD, MPH

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of

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ABSTRACT

Fresh produce can become contaminated in the field through contact with microbes in irrigation water and soil. In Georgia, many growers rely on surface water to irrigate crops even though *Salmonella* is regularly detected in surface water in this region. To identify key pathways of produce contamination and the risks of surface water irrigation, field studies were conducted in southern Georgia. *Salmonella* was regularly detected in 42.8% of surface water irrigation pond samples (*N*=507) in 2012-2013 (Chapter 1) and 33.3% of irrigation pond samples (*n*=24) in 2014 (Chapter 2). We determined that *Salmonella* could be detected not only in surface water irrigation ponds, but also in well water (20%; *n*=5), irrigation systems (26.2%; *n*=65), and even on produce (3%; *n*=65) from commercial farms (Chapter 2). These results indicate that irrigation with surface water may pose a risk to produce safety. A high level of genetic diversity (11 serovars, 17 pulsed-field gel electrophoresis patterns) was detected on these produce farms, suggesting the role of nonpoint source contamination. *Salmonella* could be detected in soil in southern Georgia and we demonstrated through field experiments that *Salmonella* in soil could be transported onto crops via splash transfer (Chapter 3). Compared to dry soils (7% soil moisture), wet soils (15% soil moisture) were associated with higher levels of *Salmonella* on produce (mean: 0.24 CFU/g vs. 0.04 CFU/g; *p*=0.04) and in splash water (at 5 cm heights: 21.14 CFU/100ml vs. 1.14 CFU/100ml; *p*=0.07). These results indicate that produce safety may be compromised by heavy rainfall events in rainy periods. Salmonellosis risk in southern Georgia was elevated by 13% following wet periods (95% CI: 6-19%). Extreme precipitation events were associated with an 11% increase in risk (95% CI: 5-18%) when they occurred in moderate rainfall or wet periods (Chapter 4). These results highlight the importance of characterizing the relationship between climatic changes, environmental reservoirs of *Salmonella*, and the pathways of environmental exposure to *Salmonella*. The findings also underscore the need for comprehensive risk assessments that can estimate the produce safety risks of irrigation and rainfall events in areas where environmental pathogens are prevalent.

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INTRODUCTION

Every year in the United States, there are an estimated 48 million cases of foodborne diseases (Scallan et al. 2011a; Scallan et al. 2011b). This means that over the course of one year, 15% of the population may become infected with a foodborne pathogen. Because the symptoms of foodborne infections often resolve on their own, many cases of foodborne infections go unreported and thus, the burden of disease may be even greater than we currently estimate. Foodborne pathogens are nondiscriminatory—anyone can become infected. And yet, these pathogens disproportionately affect the young, elderly, and the immunocompromised (Centers for Disease Control and Prevention 2017a). These populations are at greater risk of hospitalizations, and even death, from foodborne diseases.

Foodborne disease outbreaks can also be hazardous to the food industry. Upon detecting a food safety issue, manufacturers must decide whether to execute a recall of their products although ultimately, the U.S. Food and Drug Administration can mandate a recall, if necessary (Stearn 2018; U.S. Food and Drug Administration 2017). The process of removing and destroying the food product throughout the supply chain is time-consuming and costly (Grocery Manufacturers Association et al. 2010; Grocery Manufacturers Association et al. 2011). Some researchers have estimated that the average cost of a food product recall is $10 million (Grocery Manufacturers Association et al. 2010). When an outbreak can be attributed to a specific food commodity but not to a specific manufacturer, the entire sub-industry can be affected. For example, in 2006, there was a multi-state outbreak of *E. coli* O157:H7 attributed to bagged spinach. The Food and Drug Administration (FDA) promptly issued a warning about both bagged and bulk spinach. As a result, spinach was not sold, served,

harvested, or marketed for several days. The researchers at the United States Department of Agriculture Economic Research Service estimated that in the first 68 weeks after the FDA warning, retail sales of spinach fell by $201.9 million (Arnade et al. 2009). The economic impact of foodborne outbreaks is one of the many reasons that growers are key stakeholders in food safety.

Many different government entities are actively involved in the promotion of food safety in the United States. As part of the Foodborne Diseases Active Surveillance Network (FoodNet), the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), the United States Department of Agriculture, and ten state health departments collaborate to conduct active surveillance of foodborne illnesses occurring in the United States. Since its inception in 1995, FoodNet has conducted surveillance of foodborne diseases to estimate illnesses, hospitalizations, and deaths and elucidate the risk factors, sources, and trends of foodborne illnesses (Henao et al. 2015). These data are used to effect changes in industry practices and regulatory policies to improve food safety. Around the same time that FoodNet was established, the CDC and several state health departments formed PulseNet, a laboratory network that compiles molecular subtyping of foodborne pathogens (CDC 2016a). PulseNet can detect early indications of an outbreak through increases in specific pathogen subtypes added to the national PulseNet database, identify clusters of disease, and determine the source of infection by matching the outbreak subtype with the subtype isolated from the suspected food product. These capabilities allow the CDC to more efficiently conduct outbreak investigations and then disseminate critical information to minimize the public's exposure to contaminated food.

In the past two decades, due perhaps to increased surveillance efforts, there have been reductions in infections from certain pathogens, such as *Listeria* and STEC O157 (CDC 2017a). However, these strides have not been observed for infections with non-typhoidal *Salmonella*. The U.S. Department of Health and Human Services set a ten-year goal to reach 11.4 cases of salmonellosis (disease caused by *Salmonella*) per 100,000 population for their Healthy People 2020 Food Safety objective (Office of Disease Prevention and Health Promotion 2010). In 2010, incidence was approximately 15 cases per 100,000 population; in 2015, national incidence was still at 14.9 cases per 100,000 population (Centers for Disease Control and Prevention 2017b).

Salmonella infection occurs through fecal-oral transmission but exposure can occur through numerous pathways. *Salmonella* is shed through the feces of animals, including humans. Exposure to *Salmonella* can occur through direct and indirect contact with contaminated feces (Labbe 2013). Many cases are attributed to the consumption of contaminated animal-based food commodities (Jackson et al. 2013; Mughini-Gras et al. 2014; Painter et al. 2013; Pires et al. 2012) and interaction with domestic and wild animals (Hoelzer et al. 2011; Srikantiah et al. 2004; Woodward et al. 1997). Another source of infection is the consumption of fresh produce (Behravesh et al. 2011; Bowen et al. 2006; Hanning et al. 2009; Sivapalasingam et al. 2004). In a study by Painter et al., nearly half of salmonellosis incidence in the United States could be attributed to produce consumption (Painter et al. 2013). Given the public health emphasis on fresh fruit and vegetable consumption and the increasing popularity of fresh produce, it is a public health imperative to implement measures that ensure produce safety (Quested et al. 2010).

Fresh produce can become contaminated at various steps in the farm-to-fork continuum. Crops can become contaminated in the field at the preharvest level, during harvest, or in the postharvest packing, storage, and distribution steps (Bartz et al. 2017; Franz and van Bruggen 2008; Murray et al. 2017; Olaimat and Holley 2012; Park et al. 2012; Rajwar et al. 2016). Given the complexities of the food supply chain and the current geographic range of distribution, it is a challenge to conduct traceback studies when foodborne outbreaks occur. Many studies have focused on the role of postharvest processes on contamination. Following harvest, it is common for crops from various farms to be cleaned, packed, processed, and stored at the same facility until they are eventually transported and distributed locally, nationally, or even globally. Produce can become contaminated at any of these steps in the postharvest stage. Fewer studies have explored preharvest produce contamination even though several outbreaks have been traced back to on-farm conditions (Gelting et al. 2011; Greene et al. 2008). Moreover, preharvest produce contamination can introduce pathogens to facilities that process products from myriad farms (Tomás-Callejas et al. 2011). These pathogens can persist and cross-contaminate other produce commodities, thereby further complicating traceback studies.

There are numerous risk factors for preharvest contamination. Fresh produce can become contaminated through direct contact with pathogens in the soil environment of farms (Jacobsen and Bech 2012). Human pathogens may be introduced through the application of untreated biological soil amendments of animal origin (BSAAO) as fertilizer (Jung et al. 2014; Semenov et al. 2009). These soil amendments may have previously been applied to the soil of the produce field or transported through surface runoff during precipitation events from neighboring fields. The intrusion of domestic animals and wildlife on farms may also

introduce pathogens into the soil matrix (Franz and van Bruggen 2008). During heavy rainfall events, flooding and surface runoff may result in the contamination of edible portions of crops. There is also some evidence that plants may internalize pathogens through their roots (Hirneisen et al. 2012; Mootian et al. 2009; Sharma et al. 2009).

Splash during heavy rainfall events can also result in produce contamination (Boyer 2008; Cevallos-Cevallos et al. 2012; Jacobsen and Bech 2012; Monaghan and Hutchison 2012; Weller et al. 2017). The force of rain droplets can break up soil aggregates and cause soil particles to be displaced. Soil splash can result in surface sealing, which reduces water infiltration and ultimately, increases soil erosion (Assouline 2004). This has implications for increased runoff and soil pathogen transport in the environment. However, another impact of soil splash is the transport of pathogens that are adhered to soil particles. Moreover, droplet force that can disperse soil particles can also disperse planktonic (free-flowing) cells of *Salmonella*. Thus, splash water may be an effective vehicle for pathogen transfer from soil onto fresh produce.

Another major risk factor is the use of contaminated surface water or groundwater for irrigation (Decol et al. 2017; Gelting et al. 2011; Pachepsky et al. 2011; Steele and Odumeru 2004; Uyttendaele et al. 2015; Weller et al. 2015). Many growers rely on surface water ponds adjacent to fields to irrigate their crops. These surface water ponds are often created from damming streams and some are fed by groundwater. Even when groundwater from a well is accessible, growers use these ponds because of the higher withdrawal capacity. Unfortunately, many of these surface water ponds are susceptible to pollution through runoff, animal intrusion, and even human recreation (Harris et al. 2018; Levantesi et al.

2012). Even when irrigation water is free of pathogens, the intensity of water droplets during irrigation may result in the aforementioned transfer of pathogens through splash water.

The risks of *Salmonella* contamination of produce through soil, splash, and irrigation water are exacerbated by the ability of many enteric pathogens to survive in non-host environments. *Salmonella* spp. are particularly hardy—they have evolved survival strategies to withstand many environmental stressors that inactivate other pathogens (Foster and Spector 1995). They exhibit tolerance to pH fluctuations, wide ranges of heat, desiccation, osmotic stress, and nutrient limitations (Spector and Kenyon 2012; Winfield and Groisman 2003). Interestingly, the stressors can even confer greater resistance to other types of stressors and thereby potentiate the survival of salmonellae in hostile environments.

These survival mechanisms are likely employed for *Salmonella* survival in the environment. Salmonellae can survive and persist in soil for long periods of time—one study detected *Salmonella* in soil up to 332 days after inoculation (Brennan et al. 2014; Danyluk et al. 2008; Islam et al. 2004; Underthun et al. 2018; You et al. 2006). There is also evidence that given optimal conditions, *Salmonella* can experience regrowth in soil (Zaleski et al. 2005). *Salmonella* can also survive and persist in various water sources (Winfield and Groisman 2003). The survival and persistence of *Salmonella* in these non-host environments can be impacted by many abiotic and biotic factors. Some of the abiotic, or non-living physical and chemical, factors that can impact survival and persistence are soil moisture content, soil type, temperature, solar radiation, and nutrients, such as nitrates and nitrites (Erickson et al. 2014; Gu et al. 2013; Holley et al. 2006; Lang and Smith 2007; Lipp et al. 2001; McEgan et al. 2013; Pachepsky et al. 2014; Underthun et al. 2018). Biotic, or living, factors to consider are

competition and predation (Rozen and Belkin 2001; Van der Linden et al. 2013). In Chapter Three, I explore one of these parameters: the impact of soil moisture content on *Salmonella* survival and transport.

In addition to surviving in soil and water, enteric pathogens can survive and persist on the surfaces of crops (Barak and Liang 2008; Islam et al. 2005; Kisluk and Yaron 2012; Oliveira et al. 2012; Van der Linden et al. 2013). In one study, *Salmonella* Typhimurium persisted on parsley for at least four weeks and survival was mediated by temperature and sunlight (Kisluk and Yaron 2012). Given the potential for contamination of crops via irrigation water, growers often incorporate a waiting period of several days between irrigation and harvest to allow pathogen die-off to occur. However, given the evidence of the capabilities of *Salmonella* to survive in the field for several weeks, these die-off waiting periods may not be sufficient.

These pathways of produce contamination and factors underlying enteric pathogen survival in the environment are particularly relevant for growers in Georgia. In 2016, more than 39,000 hectares of fresh market vegetables—approximately $1.2 billion in value—were grown in Georgia (USDA 2017). The majority of Georgia's vegetable region is in southern Georgia, where growers use surface water for irrigation even though *Salmonella* is regularly detected in the environment (Antaki et al. 2016; Haley et al. 2009; Li et al. 2014; Maurer et al. 2015; Vereen Jr et al. 2013). Moreover, compared to other parts of the United States, Georgia consistently has higher salmonellosis incidence rates (Centers for Disease Control and Prevention 2014, 2016b, 2017b) and has higher rates of infection from serovars often detected in environmental reservoirs (e.g. wildlife and water). The high incidence rates of salmonellosis, the prevalence of *Salmonella* in the environment and the agricultural

productivity of the state make Georgia an ideal place to conduct my dissertation research on the risk factors for *Salmonella* contamination of produce.

Throughout the course of my dissertation research, the FDA worked to finalize the Produce Safety Rule of the Food Safety Modernization Act, originally signed into law in 2011. This rule was developed to address many of the major risk factors for preharvest contamination. It established science-based rules and standards to minimize the potential for the introduction of pathogens to crops. For example, water for agricultural use must be tested for generic *Escherichia coli* and the geometric mean of *E. coli* concentrations cannot exceed 126 CFU/100 ml. To comply, growers must conduct an initial survey of the water and analyze samples yearly for follow-up.

However, growers are not provided with information on when and where to sample. Growers needing to assess water quality in surface water irrigation ponds are faced with the onerous task of collecting a representative sample from these ponds. Surface water from these ponds is withdrawn via an intake pipe, which is likely the best approximation within the pond of irrigation water quality. The intake area is also often difficult to sample without a boat or specialized sampling equipment. In Chapter One, I evaluate two science-based, yet feasible shoreline-sampling strategies to find an alternative to sampling water at the intake pipe. I also assess the spatial and temporal variability of surface water quality in the study region in terms of both fecal indicator bacteria (generic *E. coli*) and human pathogenic bacteria (*Salmonella*).

In Chapter Two, I continue the study of *Salmonella* in surface water irrigation ponds to elucidate potential risks to produce safety. I investigate the presence of *Salmonella* in surface water irrigation ponds, groundwater wells, irrigation systems, and on produce to determine whether salmonellae might be moving from irrigation water sources, through irrigation systems, and ultimately onto produce. I examine the diversity and clinical relevance of *Salmonella* study isolates analyzed with serotyping, pulsed-field gel electrophoresis, and whole-genome sequencing.

In Chapter Three, I shift my focus from contaminated surface water irrigation ponds to another route of fresh produce contamination: splash water transfer. Through a series of microcosm and field experiments, I evaluate the survival of *Salmonella* in soil and the transfer of *Salmonella* from soil to produce during irrigation and rainfall events. I describe the impact of soil moisture content on *Salmonella* survival and transport to elucidate the role of antecedent soil moisture on the risks of *Salmonella* contamination of produce through splash.

The impact of antecedent moisture conditions and extreme precipitation events is explored further, but at the population level, for Chapter Four. This chapter uses data on salmonellosis in Georgia to examine the relationship between precipitation and salmonellosis incidence. The prevalence of infection with serovars associated with environmental reservoirs may suggest the importance of the contribution of *Salmonella* transmission through pathways more associated with environmental exposure in this region. Environmental exposure can be mediated by climatic factors, such as temperature and precipitation (Cann et al. 2013; Carlton et al. 2014). For example, as previously described, precipitation can result in overland flow that can transport pathogens through the environment. Precipitation can also

impact the habitats and dispersal of wildlife reservoirs (Lind 2008; Walls et al. 2013). Given the potential for climatic changes in the future, such as the increased frequency of extreme precipitation events and duration of drought conditions (Kunkel et al. 2013; Prein et al. 2016), this chapter serves to inform our understanding of the impacts various precipitation scenarios may have on infectious disease risk.

Through the first three chapters, I identify several potential routes and risk factors for *Salmonella* contamination of produce and examine the role of precipitation in mediating the prevalence of *Salmonella* in the environment. The fourth chapter is a slight departure from the produce safety focus of the previous three chapters—it extends the study of *Salmonella* in the environment to *Salmonella* infections in Georgia that may arise from a variety of environmental exposures. These four chapters represent a multidisciplinary approach to the examination of the interplay between precipitation conditions and *Salmonella* presence in the environment and ultimately, the investigation of the climatic drivers of human exposure to *Salmonella* in the environment.

CHAPTER ONE

Evaluation of Grower-Friendly, Science-Based Sampling Approaches for the Detection of *Salmonella* in Ponds Used for Irrigation of Fresh Produce

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Abstract

Recognition that irrigation water sources contribute to preharvest contamination of produce has led to new regulations on testing microbial water quality. To best identify contamination problems, growers who depend on irrigation ponds need guidance on how and where to sample these waters. In this study, we evaluated several sampling strategies to identify contamination with indicator bacteria (e.g. generic *Escherichia coli*) and pathogenic bacteria (*Salmonella* spp.*)* in five irrigation ponds used on produce farms in southern Georgia. Both *Salmonella* and *E. coli* were detected at all ponds regularly over the 19 month study period, with overall prevalence and concentrations increasing in late summer and early fall. Of 507 water samples, 217 (42.8%) were positive for *Salmonella*, with a very low geometric mean concentration of 0.05 Most Probable Number (MPN)/100 ml, and 442 (87.1%) tested positive for *E. coli*, with a geometric mean of 6.40 MPN/100 ml. We found no significant differences in *Salmonella* or *E. coli* detection rates or concentrations between sampling at the bank closest to the pump intake versus sampling from the bank around the pond perimeter, when comparing to results from the pump intake, which we considered our gold standard. However, samples collected from the bank closest to the intake had a greater level of agreement with the intake (Cohen's kappa statistic = 0.53; $p < 0.001$) than the samples collected around the pond perimeter (Cohen's kappa statistic = 0.34; $p = 0.009$) in terms of *Salmonella* presence. We also found no significant differences in *Salmonella* or *E. coli* presence between analyzing the results of three discrete samples and the result of one sample constructed by compositing aliquots from the three discrete samples. However, when using the intake pipe as the gold standard, using the results of the composited samples had a false negative rate of 47.8% while using the results of the three discrete samples had a false negative rate of 8.7%. *E. coli* concentrations were associated with increased odds of

Salmonella detection (odds ratio = 1.31; 95% CI [1.10, 1.56]) while *E. coli* presence was not associated with increased odds of *Salmonella* detection. All of the ponds would have met the Food Safety Modernization Act's Produce Safety Rule standards for *E. coli* even though *Salmonella* was also detected. Results from this study provide important information to growers and regulators about pathogen presence in irrigation ponds and inform best practices for surface water sampling. Growers should sample at the intake pipe when possible but also look into sampling water in the irrigation distribution system. They should also analyze three discrete water samples instead of compositing water samples.

Key Words: *Salmonella*, *E. coli*, Irrigation, Agriculture, On-Farm Food Safety, Food Safety Modernization Act

Introduction

In the United States, *Salmonella* causes approximately 1 million cases of foodborne illness yearly and the southeast consistently has high salmonellosis incidence rates (CDC 2014; 2016; 2017; Scallan et al. 2011b). Nationwide, nearly half of salmonellosis cases can be attributed to produce consumption (Painter et al. 2013). Crops can be contaminated with enteric pathogens, such as *Salmonella*, throughout the farm-to-fork continuum, but one important factor is preharvest contamination (Franz and van Bruggen 2008; Park et al. 2012; Tomás-Callejas et al. 2011; Wadamori et al. 2017). Preharvest contamination with enteric pathogens can occur through contact with pathogens in soil and animal feces, but also through irrigation with contaminated water (Benjamin et al. 2013; Gelting and Baloch 2013; Gelting et al. 2011; Hanning et al. 2009; Weller et al. 2015b). Surface water irrigation ponds

can become contaminated through the transport of enteric pathogens in runoff and the presence of wild animals (Park et al. 2012). The protection of irrigation water sources from contamination with enteric pathogens is thus a vital component of ensuring the production of fruits and vegetables that are safe for consumption.

In 2016, more than 39,000 hectares of fresh market vegetables—approximately $446 million in value—were grown in Georgia (USDA 2017). The majority of Georgia's vegetable growing area is in southern Georgia. One common irrigation water source is a farm pond, typically created by damming 2nd or 3rd order streams. Farm ponds are replenished by streams, surface runoff, and sometimes by groundwater from nearby wells. Even when wells are available, growers frequently irrigate directly from the pond due to the higher water withdrawal capacity.

Surface water quality can be compromised by numerous point and non-point sources of pollution, which may lead to irrigation with contaminated water (Antaki et al. 2016; Decol et al. 2017; Gelting and Baloch 2013; Gu et al. 2013; Jacobsen and Bech 2012; Jokinen et al. 2010; Weller et al. 2015b). In the southeast, *Salmonella* is regularly detected in surface waters, making farm pond use a potential produce safety concern (Haley et al. 2009; Li et al. 2014; Luo et al. 2015; Maurer et al. 2015; Strawn et al. 2014).

In 2015, the Produce Safety Rule of the Food Safety Modernization Act (FSMA; www.federalregister.gov/citation/80-FR-74353) provided growers with numerical criteria to ensure that untreated surface water applied onto produce met safety standards. However, it did not provide explicit directions for how and where to obtain water samples. Many

irrigation ponds are large, presenting a formidable challenge to growers seeking a representative water sample for analysis. Growers can choose the sampling site—this might be near the dams used to create the irrigation ponds, near the pipes where water is withdrawn from the ponds, upstream of the pond, or another more accessible location. Water upstream of the pond would provide the best approximation of the quality of water entering the pond. These water samples would demonstrate whether surface water quality was compromised by upstream activity (e.g. livestock farming). However, it would not necessarily reflect contamination events occurring at the ponds. Because of sediment deposition near the dam and the adsorption of bacteria to soil particles, bacterial concentrations might be higher near the dams than in other areas of the pond. Irrigation pumps, often located at pump stations near banks, withdraw water from ponds through intake pipes. These pipes range in length but can extend to more than 30 meters from the station to the pond. The water near the pump intake likely best represents the quality of water delivered to crops but the intake is often difficult to access from the pond's bank and sometimes accessible only by boat. Ultimately, growers and water quality analysts might select the most convenient location at the pond, but samples from this location might not reflect irrigation water quality. Thus, critical decisions regarding water sampling must be made to accurately assess agricultural water quality for compliance with the Produce Safety Rule.

To provide guidance on best sampling approaches for compliance with the Produce Safety Rule, we compared sampling strategies for testing irrigation ponds in southern Georgia. We evaluated sampling strategies that would allow growers to sample directly at the shore instead of traveling on a boat to the end of the intake pipe or using specialized sampling

equipment. In this study, we considered water at the pump intake the "gold standard" because water near this intake pipe would be most likely to ultimately enter the irrigation distribution system. We investigated differences between pump intake sampling and two shoreline sampling strategies: we sampled repeatedly from one location on the pond's shore closest to the intake (Strategy 1) and around the pond's perimeter (Strategy 2). We also examined the differences between collecting three discrete samples and consolidating three samples into one.

Additionally, even though the Produce Safety Rule criteria are for generic *Escherichia coli*, we extended our analysis to *Salmonella* spp. because prior studies by our team found *Salmonella* regularly in these ponds (Harris et al. 2018; Li et al. 2014; Li et al. 2015; Luo et al. 2015; Luo et al. 2014) and salmonellosis rates in this region are high (CDC 2017). Instead of specific pathogens, water quality assessments often use fecal indicator organisms, such as coliforms and generic *E. coli*, because they are much easier to detect in water samples. Previous studies have found a weak correlation between indicator organism and *Salmonella* prevalence in surface water (Falardeau et al. 2017; Partyka et al. 2018; Sassoubre et al. 2011) but others have found that indicator organisms have the potential to predict *Salmonella* presence (Efstratiou et al. 1998; Partyka et al. 2018). These contradictory results make it difficult to conclude whether the Produce Safety Rule standards can sufficiently assess agricultural water quality. Moreover, the capacity for the use of *E. coli* as an indicator may vary by location and thus, a study of both *E. coli* and *Salmonella* in southern Georgia is warranted to characterize the microbiological quality of agricultural water in the produce production environments of this region. The objectives of this study were to examine the spatial variability of *E. coli* and *Salmonella* prevalence and concentrations across and within five ponds, the temporal

variability in *E. coli* and *Salmonella* prevalence and concentrations, and several sampling strategies for growers to implement in southern Georgia.

Materials and Methods

Sample Collection

We sampled five irrigation ponds on commercial mixed-use produce farms monthly from March 2012-September 2013. These ponds ranged in size from 0.6-8.7 hectares and spanned a wide area of southern Georgia as indicated in Figure 1-1. Because of the large distances between farms, ponds were visited monthly but during separate weeks, with two ponds (CC2 and MD1) sampled during the first week of the month, and the remaining three ponds (NP, LV, SC1) sampled during the third week. Each pond was visited 19 times over the study period. At each visit, we sampled the water surface directly above the pump intake, which we considered the "gold standard" for the microbiological quality of water applied onto crops because it was where water entered the irrigation distribution system (Figure 1-2). The sampling area near the intake represented the area of the pond most proximal to the irrigation distribution system and thus, the water in this area would most likely be withdrawn during irrigation. The area near the intake pipe was also the location of sampling for prior studies on irrigation water quality in this region (Antaki et al. 2016; Harris et al. 2018; Li et al. 2014). We evaluated two shoreline sampling strategies to provide growers with information on where to sample in the ponds. Due to sampling feasibility, shoreline sampling strategies were alternated between months. For Strategy 1, three 4.5 L grab samples, approximately 3 m apart, were collected at the edge of the pond near the intake. For Strategy 2, 4.5 L grab samples were collected at three fixed, easily accessible locations along the perimeter of the pond: at the bank near the intake, on the pond dam, and a third point equidistant from the

other two locations. The three sampling points were selected to represent the landscape around the perimeter.

From March-September 2013, for both strategies, in addition to the intake sample at the water surface, a sample was collected from 0.5 m below the surface, closer to the pipe intake. Sampling strategies are depicted in Figure 1-3. A total of 507 samples were collected. During three sampling events, water levels at Pond CC2 were low so we were unable to collect the full set of samples. In one event for Strategy 2, only two of the three edge samples were collected. In the other two events (Strategy 1 and 2), the subsurface sample could not be taken.

In addition to evaluating where to sample, we also evaluated differences in the method of sampling. In particular, we were interested in evaluating the number of edge samples necessary to adequately describe water quality at the intake pipe. For our sampling strategies, we sampled at the edge in triplicate but were interested in evaluating an alternative to collecting three samples and so we evaluated differences between collecting and analyzing three discrete samples and compositing these three samples into one sample for analysis. To do so, for both strategies, in addition to collecting three discrete grab samples at the shore, equal volumes from these grab samples were combined to create a composite 4.5 L sample.

Immediately following collection, samples were placed on ice and brought to the laboratory for analysis within 24 hours. During sampling, a YSI model 6920 multi-probe data sonde (YSI Incorporated, Yellow Springs, Ohio, USA) was used to measure pH, temperature, dissolved oxygen concentration, turbidity, and specific conductivity. A model TR-5251

tipping bucket rain gauge (Texas Electronics, Dallas, Texas, USA) with a Hobo Pendant® event data logger (Onset, Bourne, Massachusetts, USA), installed on the bank near the pump, recorded precipitation events.

Sample Analysis

Salmonella concentrations were enumerated using a culture-based most probable number (MPN) method (Luo et al. 2014). Briefly, three dilutions of water samples were pre-enriched with 2x lactose broth (Becton, Dickinson and Company (BD) Difco™, Franklin Lakes, NJ) and then selective tetrathionate broth with iodine (Thermo Scientific™, Remel™, Lenexa, KS). Cultures were streaked onto xylose-lysine-Tergitol-4 (BD) and CHROMagar™ *Salmonella* Plus (CHROMagar™ Microbiology, Paris, France) agar. Presumptive positives were confirmed using PCR targeting the *invA* gene (Chiu and Ou 1996). Samples were analyzed for total coliform and *E. coli* using the Quanti-Tray/2000 System with Colilert Reagent (IDEXX Laboratories, Westbrook, ME). The physicochemical parameters of NO_3-N, NH_4-N, total nitrogen, PO_4-P, total phosphorus, Cl^-, and total suspended solids were measured using colorimetric autoanalyzers.

Statistical Analysis

Concentrations below the limit of detection (LOD; *Salmonella*: 0.0548 MPN/100 ml; *E. coli*: 1 MPN/100 ml) were assigned a value of half the LOD. Concentrations above the upper LOD (*Salmonella*: 11 MPN/100 ml; *E. coli*: 2419.6 MPN/100 ml) were set to the LOD. To address skewness, we log-transformed *Salmonella* and *E. coli* concentrations. For presence/absence analysis, concentrations below the LOD were considered to have an absence of *Salmonella* or *E. coli*.

Temporal and spatial differences in concentrations and proportion of samples positive were assessed. Pearson's chi-squared tests were used to compare the proportion of samples positive in each strategy, irrigation pond, and month. Tukey Honest Significant Difference tests were used to analyze differences in mean concentrations at the 95% overall confidence level between strategies, irrigation ponds, and months for *Salmonella* and *E. coli*.

The strategies (shoreline sampling near the intake vs. shoreline sampling around the perimeter; three discrete samples vs. one composited sample) were evaluated by comparing *Salmonella* and *E. coli* presence in the edge and composite samples to the intake ("gold standard"). One method for comparing these two strategies was to estimate the level of agreement between edge/composite samples and intake samples. For each strategy, in terms of *Salmonella* and *E. coli* presence/absence, we calculated the percentage of edge and composite samples that matched the intake sample. This percentage reflected the level of agreement between edge/composite and intake for each strategy, with higher percentages indicating higher levels of agreement. Another method for comparing strategies was through calculating the Cohen's kappa statistic for *Salmonella* and *E. coli* presence (Cohen 1960). Similar to the previous method, edge and composite samples were compared to the intake. Instead of comparing individual edge samples to the intake, edge sample data were aggregated for each strategy. More specifically, *Salmonella* or *E. coli* detection in any edge sample rendered the overall edge sample positive for *Salmonella* or *E. coli*. The third method of comparing strategies was to determine the level of pond misclassification that would occur for each strategy with the assumption that the intake sample accurately reflected the overall quality of the pond. We calculated the percentage of edge and composite samples

that would have classified the pond as *Salmonella*/*E. coli*-positive even though the intake sample was negative ("false positive") using the formula: # of "false positives" / (# of "false positives" + # "true negatives"). We calculated the percentage of edge and composite samples that would have classified the pond as *Salmonella*/*E. coli*-negative even though the intake sample was positive ("false negative") using the formula: # of "false negatives" / (# of "false negatives" + # of "true positives"). The percentage of "false positives" and "false negatives" were calculated for individual and aggregated edge samples. Thus, in addition to comparisons between Strategy 1 and 2, comparisons were also made between the misclassification potentials of the individual edge vs. aggregated edge results.

The associations between potential biological, physical, and chemical predictors of water quality and *Salmonella* presence were estimated by fitting a logistic regression model with random effects for pond. Both $\log_{10}$-transformed *E. coli* concentrations and *E. coli* presence were considered biological predictors of *Salmonella* presence because of prior studies demonstrating the relationship between *E. coli* and *Salmonella* (McEgan et al. 2013; Partyka et al. 2018). The physical and chemical parameters used were: water turbidity in Nephelometric Turbidity Units (NTU), total suspended solids (mg/l), specific conductivity in micro-Siemens per centimeter (µS/cm), oxidation-reduction potential (ORP; mV), pH, and temperature (°C). These parameters were also selected in accordance with parameters used by McEgan et al (McEgan et al. 2013). In all models, temporal effects were controlled for with a variable for month. Statistical analyses were performed in R 3.1.3 (R Core Team 2015).

Scenario Testing for Study Ponds

While this study was conducted prior to the issuance of the finalized Produce Safety Rule, we examined the hypothetical scenario of testing study ponds for FSMA compliance. FSMA requires growers to conduct an initial survey of *E. coli* in agricultural water. In this survey, the geometric mean (GM) of concentrations in at least 20 samples must be less than 126 Colony Forming Units (CFU)/100 ml. From their measurements, growers must also calculate the statistical threshold value (STV), which approximates the 90th percentile of a normal distribution (z-score = 1.28) of *E. coli* values. The STV must not exceed 410 CFU/100 ml.

For each pond, we calculated the GM and STV in two ways: 1) using all *E. coli* values from the study period and 2) selecting 20 of the highest *E. coli* values over the study period to simulate a worst-case scenario. In addition to comparing the GMs and STVs to FSMA criteria, we determined the number of samples whose indicator results disagreed with pathogen results. We refer to samples that were *Salmonella*-positive even though *E. coli* levels were below the standard as "false negatives" and samples that were *Salmonella*-negative even though *E. coli* levels were above the standard as "false positives." For ponds in compliance with the standards, we calculated a false negative rate using the formula: (# of samples positive for *Salmonella*) / (# of samples). For ponds that did not meet the standards, we calculated a false positive rate using: (# of samples negative for *Salmonella*) / (# of samples).

Results

Two hundred seventeen (42.8%) samples were *Salmonella*-positive, with a GM of 0.06 MPN/100 mL (STV: 0.25 MPN/100 ml). Four hundred forty-two (87.1%) were *E. coli*-positive, with a GM of 6.40 MPN/100 ml (STV: 61.4 MPN/100 ml). A summary of microbiological water quality results can be found in Table 1-1.

There were no significant differences in *Salmonella* concentrations or proportion of samples positive between the intake, edge, and composite samples (Figure 1-4). This was also true for *E. coli* (Supplementary Figure S1-1). Strategies had similar levels of agreement between *Salmonella*/*E. coli* presence/absence in edge, composite, and intake samples (Table 1-2). However, agreement levels were slightly higher for Strategy 1 than for Strategy 2.

When comparing *Salmonella* presence in edge vs. intake samples using the kappa statistic, Strategy 1 edge samples had a kappa of 0.53 (p <0.001) while Strategy 2 edge samples had a kappa of 0.34 (p = 0.009). Higher kappa values indicate higher levels of agreement: statistics between 0.21-0.40 indicate fair agreement while those between 0.41-0.60 indicate moderate agreement (Landis and Koch 1977). Thus, Strategy 1 edge samples showed moderate agreement with the intake while Strategy 2 edge samples showed fair agreement. When comparing composite samples, the opposite was true; Strategy 1's composite had a value of 0.38 (fair; p = 0.005) and Strategy 2's, 0.42 (moderate; p = 0.005). Ultimately, Strategy 1 edge samples had the highest kappa statistic for consistency of *Salmonella* results. When comparing strategies in terms of the consistency of *E. coli* results, Strategy 1 edge samples had a kappa of -0.04 (p = 0.64) and the composite samples had a kappa of 0.41 (p = 0.004). Strategy 2 edge and composite samples were more consistent with the intake—edge and composite samples had kappa statistics of 0.56 (p < 0.001) and 0.49 (p < 0.001), respectively.

For both shoreline sampling strategies, individual edge samples and composited samples (combining aliquots of three discrete edge samples into one sample) generally had similarly high rates of "false positive" (11.1-50.0%) and "false negative" (27.3-56.5%)

misclassifications of the intake in terms of *Salmonella* presence (Table 1-3). In contrast with the *Salmonella* results, in terms of *E. coli*, the edge and composite samples of both sampling strategies had lower "false negative" rates of 2.4-7.3%. Combining the results of multiple edge samples—if at least one edge sample were positive, all edge samples would be considered positive—reduced disagreement with the intake in terms of "false negatives" for both *Salmonella* (8.7-9.1%) and *E. coli* (0-2.4%). Strategy 1 performed marginally better than Strategy 2 in terms of lowering the rate of *Salmonella* "false negatives" (8.7% vs. 9.1%) but for *E. coli*, the opposite was true (0% vs. 2.4%). For both sampling strategies, grouping the edge results for *Salmonella* and *E. coli* resulted in false positive rates that were higher than the false positive rates from evaluating each edge sample individually or as a composited sample.

There were significant differences in *Salmonella* concentrations amongst the ponds (Figure 1-5; Supplementary Table S1-1). CC2 had the highest mean concentration of *Salmonella*, significantly higher than two other ponds (NP: difference = 0.74 log MPN/100 ml, p adj <0.001; LV: difference = 0.49 log MPN/100 ml, p adj = 0.015). NP had the lowest concentration, significantly lower than three ponds ((CC2: see above; MD1: difference = 0.64 log MPN/100 ml, p adj <0.001; SC: difference = 0.48 log MPN/100 ml, p adj = 0.018). There were also significant differences in the proportion of samples positive for *Salmonella* (χ^2 = 14.7935, df = 4, p-value = 0.005). Universally, ponds with higher proportions of samples positive for *Salmonella* also had higher concentrations of *Salmonella*. CC2's *E. coli* levels were significantly greater than levels at the other ponds (maximum difference between MD1 and CC2: 1.37, p adj <0.001).

Seasonality was evident in this study, with concentrations and proportions of samples positive increasing in late summer and early fall. *Salmonella* concentrations peaked in October and the proportion of samples positive for *Salmonella* peaked in September (Figure 1-6). The seasonal pattern for *E. coli* differed slightly from that of *Salmonella*; the peak of concentrations occurred in July and *E. coli* was regularly detected in more than half of study samples (Supplementary Figure S1-2).

Log_{10}-transformed *E. coli* concentrations were associated with increased odds of *Salmonella* detection. A 1-log increase in *E. coli* concentration was associated with a 31% increase in the odds of *Salmonella* presence (odds ratio = 1.31; 95% CI [1.10, 1.56]). On the other hand, *E. coli* presence was not associated with increased odds of *Salmonella* detection (odds ratio = 0.94; 95% CI [0.37, 2.39]). None of the chemical and physical parameters was associated with *Salmonella* presence. Odds ratios for independent variables can be found in Supplementary Table S1-2.

At all ponds, *E. coli* geometric means never exceeded 126 CFU/100 ml (highest GM: 14.91 MPN/100 ml at CC2) and the STVs met FSMA standards (highest STV: 67.5 MPN/100 ml at LV). When limiting our analysis to the samples with the highest *E. coli* levels (worst-case scenario), two ponds (CC2 and LV), had GMs greater than 126 CFU/100 ml. These two ponds also had STVs exceeding the standard (LV: 693.8 MPN/100 ml; CC2: 747.1 MPN/100 ml).

In this hypothetical worst-case scenario, three ponds (MD1, NP, and SC) would have met FSMA standards, yet we detected *Salmonella* in all ponds. Deeming these ponds *Salmonella*-

negative based on *E. coli* concentrations would have yielded "false negative" results in 80% of MD1 samples, 100% of NP samples, and 60% of SC samples. In contrast, the remaining ponds (CC2 and LV) had GMs exceeding FSMA standards, but this would have resulted in "false positive" results in 35% of CC2 and 25% of LV samples. The sensitivity (true positive rate) and specificity (true negative rate) of using *E. coli* presence as an indicator of *Salmonella* presence is 7.4% and 96.6%, respectively.

Discussion

In every month of sampling, *Salmonella* was detected—at low concentrations—in at least one of the irrigation ponds. Nearly 90% of pond samples had detectable *E. coli* and increases in *E. coli* concentrations were associated with increased odds of detecting *Salmonella.* All of our study ponds would have been considered safe for agricultural use in terms of FSMA's *E. coli*-based standards; however, *Salmonella* was detected at all ponds.

Sampling Approaches

In our study ponds, pump intakes were 3-6 meters from pond banks, at depths of 1-2 meters, and so growers would need boats or specialized equipment for sampling. When comparing two shoreline sampling strategies in terms of *Salmonella* concentrations and prevalence, we found no statistical difference between sampling near the intake (Strategy 1) and sampling around the pond perimeter (Strategy 2). This is similar to a study by Hilton et al. (1989) that found no difference in nutrient concentrations between five sampling techniques (e.g. sampling at the deepest point of the lake vs. near the water's edge). In a study by Partyka et al. (2018), spatial variability of fecal indicator bacteria was site-specific—the horizontal location explained nearly all of the variance for one reservoir and non-

significant and uninformative for another reservoir. Thus, in the present study, location within study ponds may not sufficiently account for differences in *Salmonella* and *E. coli* prevalence and concentrations.

Even so, there was correspondence among edge samples collected near the intake and between the edge and intake samples, which suggests that Strategy 1 may be marginally better at approximating the quality of water pumped through the distribution system. This was likely due to the proximity of Strategy 1 sampling sites to the intake. Within a pond, *Salmonella* and *E. coli* concentrations can vary by location depending on differences in factors known to influence bacterial survival, such as temperature, nutrients, and oxygen (Yu et al. 2014a; Yu et al. 2014b). Sampling sites closer together would likely share these survival-promoting factors. The potential spatial variability in these factors within each pond could also explain the differences observed between bank sampling at the intake and bank sampling at other parts of the pond.

The variability in water quality in the irrigation ponds might be due to the localization of *Salmonella* and *E. coli* within the pond. These variations in water quality around the pond may reflect the formation of biofilms in certain areas of the pond. There is evidence that there may be differential microhabitat and physicochemical preferences for *Salmonella* and *E. coli* in surface water (Mugnai et al. 2015; Partyka et al. 2018). Higher concentrations of *Salmonella* and *E. coli* were found by Mugnai et al. (2015) on lateral sides of pools and areas where sand accumulated but *Salmonella* was found at greater depths. *Salmonella* was positively correlated with temperature and negatively correlated with oxygen levels but the opposite was true for *E. coli*. Depending on the homogeneity of the various areas of the pond, these preferences

for location and physicochemical factors may have resulted in different spatial distributions of *Salmonella* and *E. coli*. Future studies of surface water quality should examine these preferences further and elucidate spatial patterns of indicator organism and pathogenic organism prevalence. Better understanding the spatial differences between indicators and pathogens can help inform the improvement of water sampling strategies.

It is important to note that while results show that bank sampling near the intake is often a good representation of pathogen presence at the intake, it may not be a reliable indicator of intake water quality—the "false positive" rate for *Salmonella* when grouping Strategy 1 edge samples was 37%. Thus, neither of the shoreline sampling strategies may adequately reflect water quality at the intake. Again, this may be due to the spatial heterogeneity of the microbial communities at different areas of the pond. For both strategies, considering the results from all three edge samples reduced the "false negative" rate compared to evaluating any one of the individual edge or composited samples.

This study used water quality at the intake pipe as the "gold standard" for irrigation water quality. However, it is important to note that the intake may not accurately reflect the quality of water applied to the field or even the overall quality of the pond, only water quality at the intake at one moment in time. This is further supported by the spatial heterogeneity we found in microbial contamination at each pond. In prior studies of indicator bacteria distribution in surface water, there was also evidence of spatial differences in concentrations (Davis et al. 2005; Jenkins et al. 2012; Pachepsky et al. 2018). Given the differences Pachepsky et al. (2018) also found in the interior of the pond vs. the bank, it may be useful for growers to collect samples from both the bank and the interior of the pond in order to

capture this spatial variation. To determine the suitability of surface water for agricultural use, it may be more informative for growers to analyze water in the irrigation distribution system than in the irrigation ponds because of the difficulty of collecting a representative sample from these ponds but also because there is evidence that biofilms can form within the intake pipe (Blaustein et al. 2015; Pachepsky et al. 2012). The presence of biofilms in the intake pipe could result in irrigation water contamination even when surface water is deemed suitable for irrigation. Ideally, monitoring indicator bacteria at both the pond and within the irrigation distribution system could help growers identify the area where contamination may be entering the system.

Seasonality

The increased prevalence and concentrations of *Salmonella* observed in the late summer and early fall in this study are consistent with other studies in this region (Antaki et al. 2016; Haley et al. 2009; Li et al. 2014). Our results show that *E. coli* levels spike in early- to mid-summer, concurrent with peak temperatures, whereas *Salmonella* concentrations peaked in early fall. This peak in the early fall indicates that *Salmonella* concentrations might not be similarly correlated with temperature. One study of *Salmonella* in soil observed a large initial decline in *Salmonella* concentrations when *Salmonella* was applied to soil in simulated summer conditions and a lower rate of decline and lengthy survival period when *Salmonella* was applied to soil in simulated fall conditions (Holley et al. 2006). Similarly, other studies have observed longer *Salmonella* survival in soil when temperatures are lower than 30°C (Semenov et al. 2007; Underthun et al. 2018). It might also indicate differences in *Salmonella* loading into surface water through increased rainfall (Haley et al. 2009; Jokinen et al. 2010). Salmonellosis incidence—and in turn, *Salmonella* shedding—increases in the summer and fall

(CDC 2017b). There is evidence that leakage from septic tanks can be an important driver of watershed pollution (Sowah et al. 2014; Verhougstraete et al. 2015); once present in the environment through septic leakage, *Salmonella* can then transported into surface water via runoff and leaching. Thus, the combination of increased precipitation and increased shedding in the summer months might result in the subsequent contamination of surface water in the fall months. The peak might also be explained by the increased activity of animal reservoirs of *Salmonella* in this region (Srikantiah et al. 2004).

Indicators of Microbial Contamination

Numerous previous studies have shown that *E. coli* may be a poor indicator of *Salmonella*; there is little to no correlation between *E. coli* and *Salmonella* concentrations (Haley et al. 2009; McEgan et al. 2013) as well as between *E. coli* and *Salmonella* presence (Ahmed et al. 2010; Cerna-Cortes et al. 2013; Wilkes et al. 2009). However, we found that *E. coli* concentrations were associated with increased odds of detecting *Salmonella*, which is consistent with several other studies of *Salmonella* and *E. coli* (McEgan et al. 2013; Partyka et al. 2018).

The *E. coli* and *Salmonella* concentrations in the present study are similar in magnitude to the concentrations detected in a previous study of surface water quality in southern Georgia by Antaki et al. (2016) and one order of magnitude less than concentrations in a study by Haley et al (2009). These results provide further evidence of consistent contamination of surface water in this region with *Salmonella*. In southern Georgia, the prevalence of *Salmonella* in the environment—even when *E. coli* levels are low—suggests that some sources of agricultural water that meet the Produce Safety Rule standards can still harbor pathogens. Similarly, in a

study of surface water in Florida by Topalcengiz et al. (2017), *E. coli*-based Produce Safety Rule standards could not ensure that agricultural waters did not contain *Salmonella* and STEC. In a literature review of indicator-pathogen relationships conducted by Pachepsky et al. (2016), many studies found a statistically significant relationship between *Salmonella* and indicator organisms but the correlation was often weak.

One possible explanation for the weak correlation between *Salmonella* and *E. coli* might be that the sources of *Salmonella* in this region are different from the main sources contributing to generic *E. coli* levels (Pachepsky et al. 2016). Because generic *E. coli* can be naturally prevalent in soil (Nautiyal et al. 2010), precipitation events can result in overland flow, which can transport generic *E. coli* into surface water sources (Jokinen et al. 2010; Rodgers et al. 2003; Topalcengiz et al. 2017). If the main contributor to *Salmonella* contamination of a pond were wildlife activity directly with the pond (Jokinen et al. 2011; Maurer et al. 2015), then high levels of *E. coli* following a rainfall event would not necessarily be associated with *Salmonella* levels.

Another possible explanation is that because *Salmonella* concentrations in surface water in this region are low, the grab sample volumes collected in the present study may have been too small to detect pathogens at low concentrations. In a study by Benjamin et al. (2013), Moore swab samples (cheesecloths that were immersed in surface water for several days) were used in addition to grab samples. Unlike grab samples, Moore swab samples cannot be used to quantify pathogen concentrations but because these swabs can come into contact with greater volumes of water, it can be used when pathogen concentrations are low or when pathogens are not evenly distributed in a pond. Benjamin et al. (2013) detected higher

prevalence of *E. coli* O157:H7 with Moore swabs than with grab samples. These results suggest that the grab samples used in the present study may not have been able to adequately reflect *Salmonella* contamination at the farm ponds.

However, in the same study by Benjamin et al. (2013), *E. coli* O157:H7 and *Salmonella* were detected in both grab samples and Moore swabs even though pre-irrigation water met generic *E. coli*-based standards for Good Agricultural Practices. In a study of *Salmonella* in surface water in Georgia by Haley et al. (2009), 2 L of water was collected for analysis and *Salmonella* was detected in samples that both met and exceeded single sample standards for generic *E. coli*. In another study by Partyka et al. (2018), 20 L surface water samples were concentrated to 500 ml and even then, generic *E. coli* was unable to predict the presence of *Salmonella*. Even when larger volumes of water were sampled in these studies, the *E. coli*-based standards were inadequate in ensuring that agricultural water did not contain enteric pathogens.

Conclusions

Our findings suggest that *Salmonella* and *E. coli* concentrations vary widely within each pond but that shoreline sampling near the intake may be an adequate alternative to sampling at the intake. Using the aggregated results of three individually analyzed edge samples is also better at reflecting *Salmonella* (pathogen) and generic *E. coli* (indicator) presence at the intake than using the results of analyzing one composite sample created from aliquots of the three edge samples. We detected pathogenic bacteria in ponds used for produce irrigation but the health risk posed to consumers is unclear given the low concentrations detected. While the Produce Safety Rule may be appropriate in regions where *Salmonella* is not regularly detected in the

environment, future studies focusing on the risks of irrigating with surface water in the southeast—where *Salmonella* and salmonellosis levels are high—will be crucial in providing science-based improvements to FSMA to promote produce safety nationwide.

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Figure 1-1: Map of the study region in Georgia. The study took place in the southern region of the state of Georgia. The geographic region within which the five ponds were located is indicated by the rectangle.

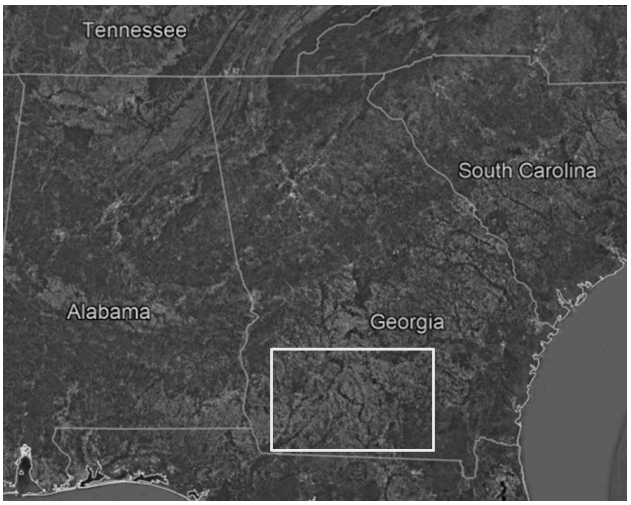


Figure 1-2: Collecting samples at the pump intake of pond NP. The screened intake at the end of the intake pipe is suspended between 1-2 m below the surface with the support of a plastic drum. The inset shows the intake.

Figure 1-3: Example of sampling strategies at one pond. All sampling events included a sample near the intake of the pump (star), which served as the gold standard for comparison. For Sampling Strategy 1, three grab samples were collected from the edge of the pond, near the intake of the pump. For Sampling Strategy 2 three grab samples were collected from the edge of the pond, around the pond's perimeter.

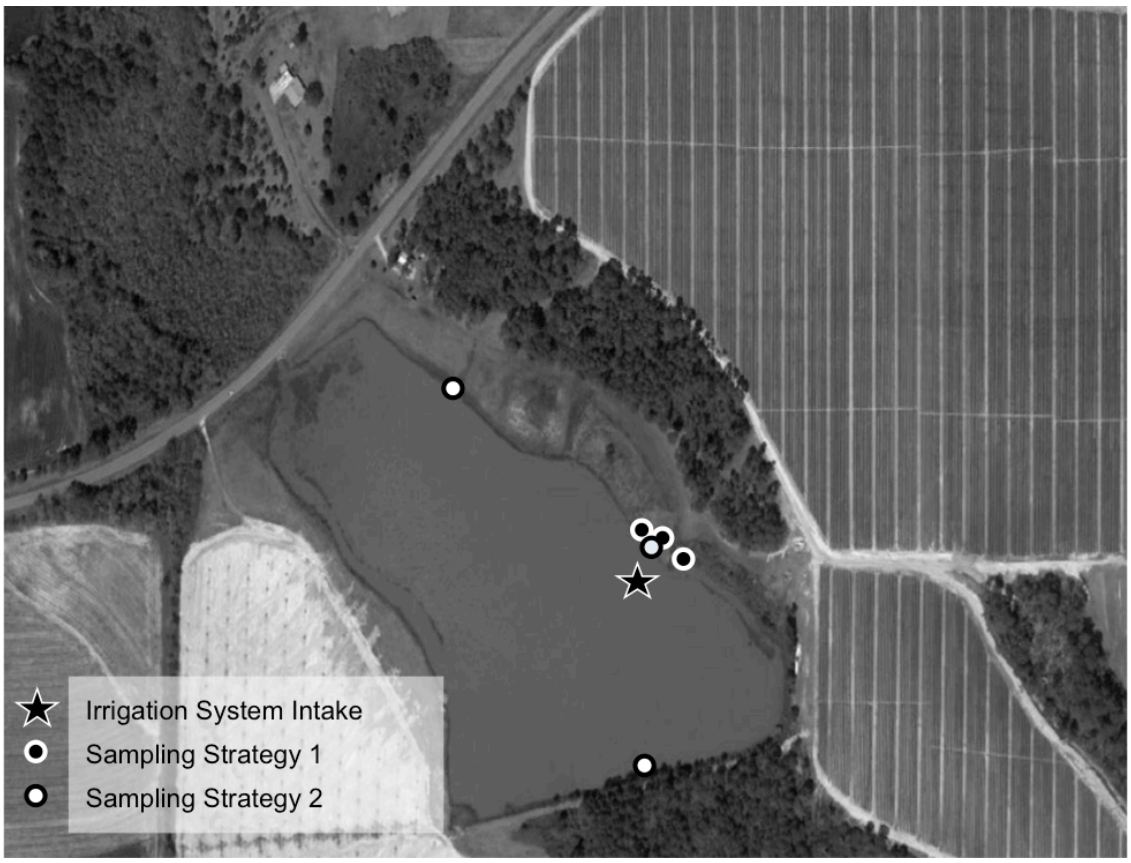


Figure 1-4: Comparison of *Salmonella* spp. concentrations by sampling strategy, stratified by the different sample types within each strategy. The bar plot compares the geometric mean of *Salmonella* concentrations (left y-axis) for each sample type. "Comp" corresponds to the composite sample, created by combining aliquots of the three edge samples. "Intake" refers to the sample collected at the surface of the pond directly above the intake pump (considered the gold standard) while "subs" samples were collected below the surface of the pond but above the intake pump. For each strategy (differentiated by color: white for strategy 1 (bank sampling closest to the intake), grey for strategy 2 (bank sampling at three locations around the perimeter of the pond), three edge samples (a, b, c) were collected. Composite, edge, intake, and subsurface samples are numbered according to their associated strategy (1 or 2). Error bars represent the standard error around the geometric mean. The scatter plot of open circles compares the proportion of positive samples (right y-axis) for each sample type. Sample sizes are indicated at the bottom of each bar.

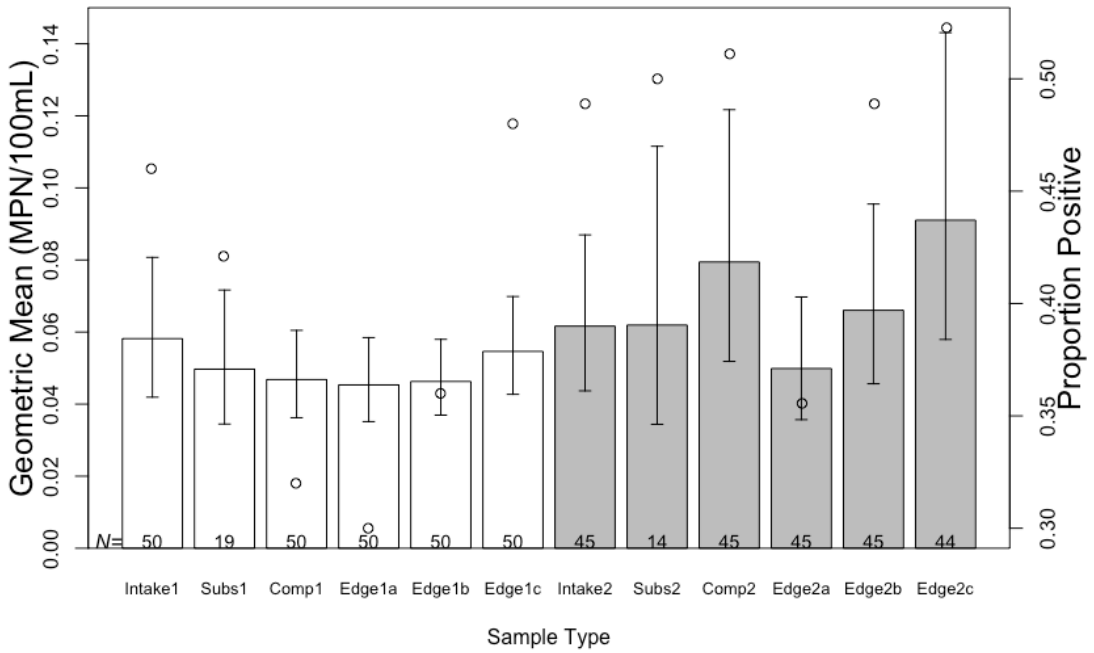


Figure 1-5: Comparison of *Salmonella* spp. concentrations (bar plot; left y-axis) and proportion of samples positive for *Salmonella* (open circles; right y-axis) amongst the five ponds in this study (CC2, LV, MD1, NP, SC). Significant differences between geometric means of individual ponds are indicated below the plot.

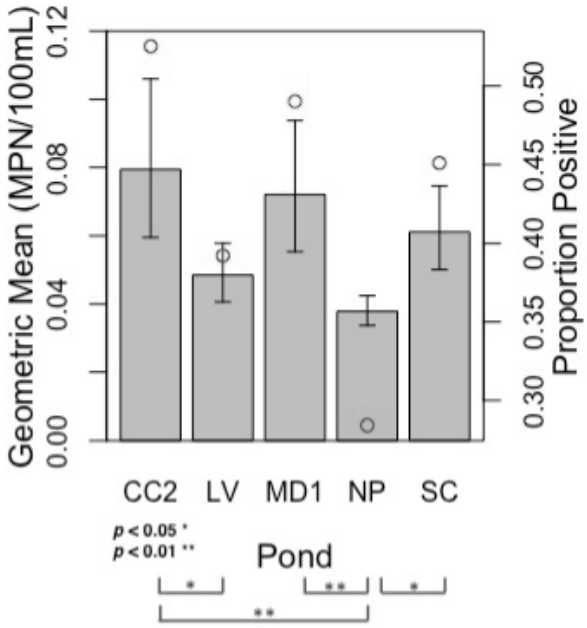


Figure 1-6: Seasonal trend of *Salmonella* spp. concentrations (bar plot; left y-axis) and proportion of samples positive for *Salmonella* (open circles; right y-axis) during the 19-month study period (March 2012 to September 2013). Sample sizes are given at the base of the bars.

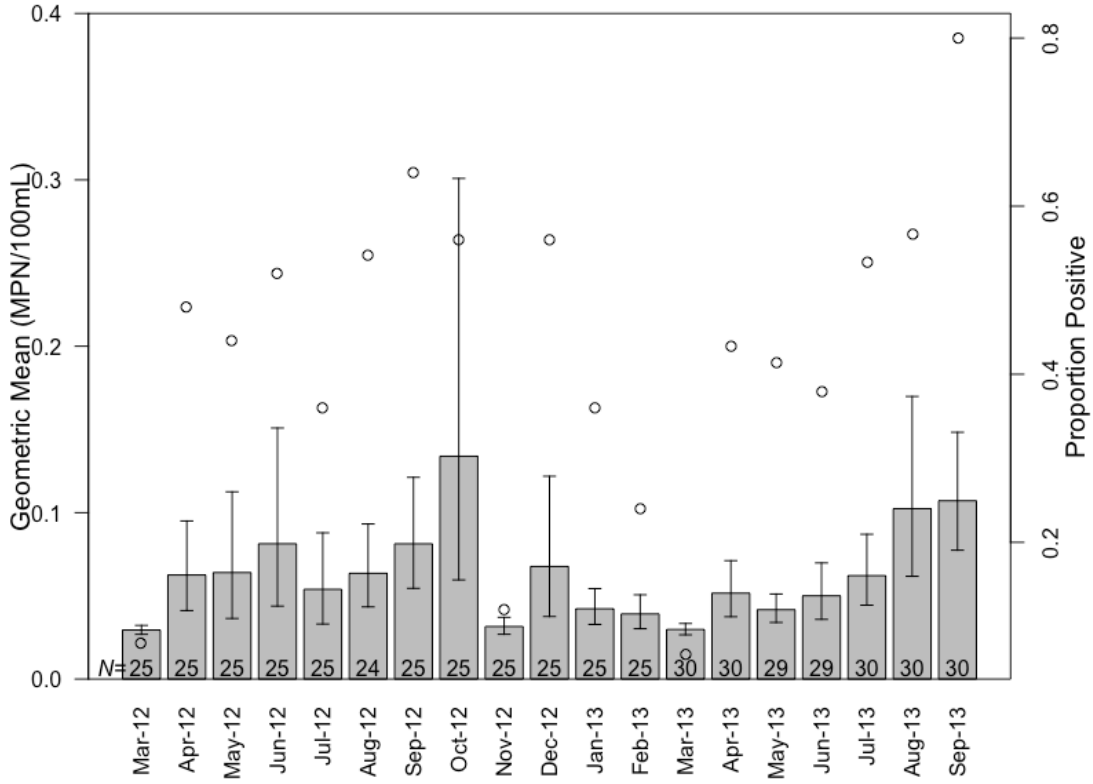


Table 1-1: Summary of *Salmonella* spp. and *Escherichia coli* concentrations (geometric mean and 95% confidence interval) and proportion of samples positives for *Salmonella* or *E. coli* by site.

		Salmonella		*E. coli*	
Pond	*N*	Geometric Mean (95% Confidence Interval)	Percent Positive	Geometric Mean (95% Confidence Interval)	Percent Positive
CC2	99	0.08 (0.06, 0.11)	53	14.91 (10.69, 20.80)	96
LV	102	0.05 (0.04, 0.06)	39	5.02 (3.39, 7.44)	83
MD1	102	0.07 (0.06, 0.09)	49	3.78 (2.66, 5.37)	71
NP	102	0.04 (0.03, 0.04)	28	6.15 (4.66, 8.13)	91
SC	102	0.06 (0.05, 0.07)	45	6.31 (4.68, 8.49)	95
Total	507	0.06 (0.05, 0.06)	45	6.40 (5.49, 7.46)	87

Table 1-2: Presence/Absence Agreement for (a) *Salmonella* spp. and (b) *Escherichia coli*. Samples were compared to others within the same strategy, e.g., Intake 1 was compared to Composite 1 and Edge 1 while Intake 2 was compared to Composite 2 and Edge 2. Sampling strategies are described in the text.

a) *Salmonella*

	Composite	Intake
Intake 1	70.0%	--
Intake 2	71.1%	--
Edge 1 (grouped)	70.0%	76.0%
Edge 2 (grouped)	64.4%	66.7%

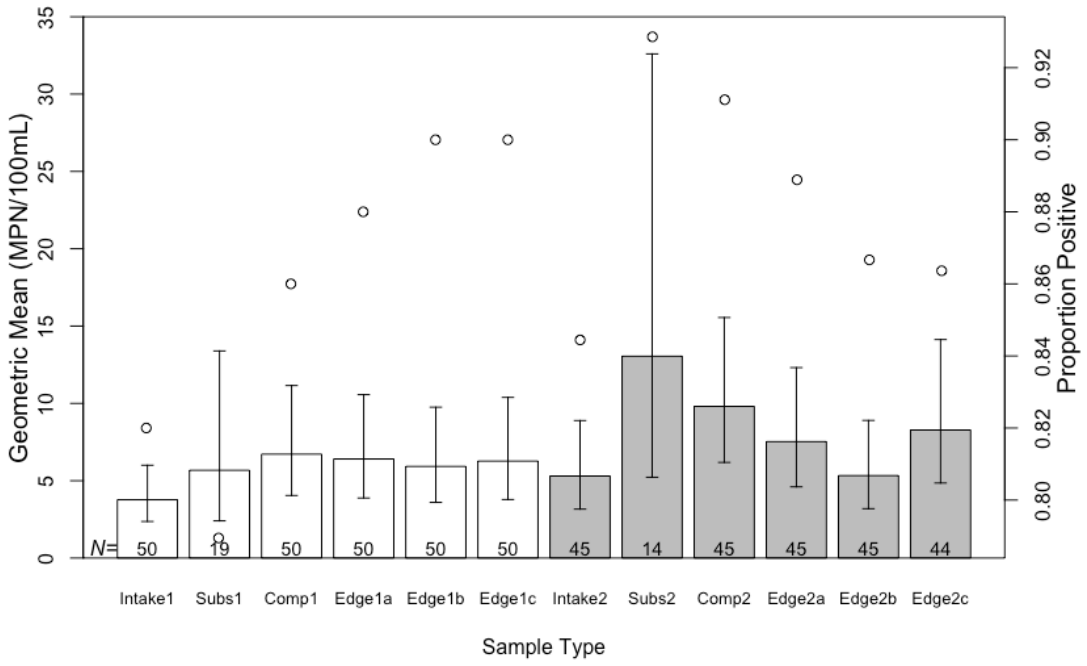
b) *E. coli*

	Composite	Intake
Intake 1	84.0%	--
Intake 2	88.9%	--
Edge 1 (grouped)	88.0%	80.0%
Edge 2 (grouped)	93.3%	91.1%

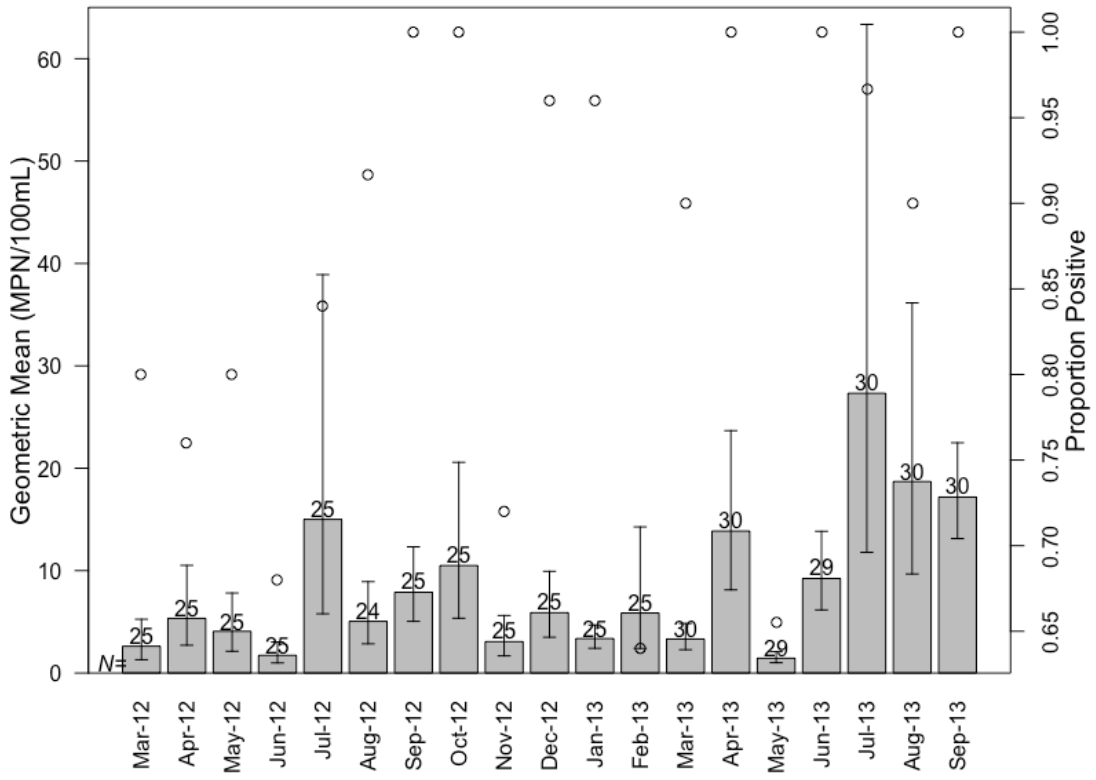
Table 1-3: Performance of sampling strategies compared to the intake (gold standard) in detecting *Salmonella* spp. and *Escherichia coli*. Sampling strategies are described in the text. Edge 1 (grouped) and Edge 2 (grouped) represent whether any of the three edge (a, b, c) samples were positive for *Salmonella*/*E. coli*.

	Salmonella		*E. coli*	
	False Negatives	False Positives	False Negatives	False Positives
Composite 1	47.8%	14.8%	7.3%	55.6%
Edge 1 (grouped)	8.7%	37.0%	2.4%	100.0%
Edge 1a	47.8%	11.1%	2.4%	44.4%
Edge 1b	56.5%	18.5%	2.4%	55.6%
Edge 1c	34.8%	33.3%	7.3%	77.8%
Composite 2	27.3%	30.4%	2.6%	57.1%
Edge 2 (grouped)	9.1%	56.5%	0%	57.1%
Edge 2a	45.4%	17.4%	2.6%	42.9%
Edge 2b	31.8%	30.4%	5.3%	42.9%
Edge 2c	45.5%	50.0%	5.4%	42.9%

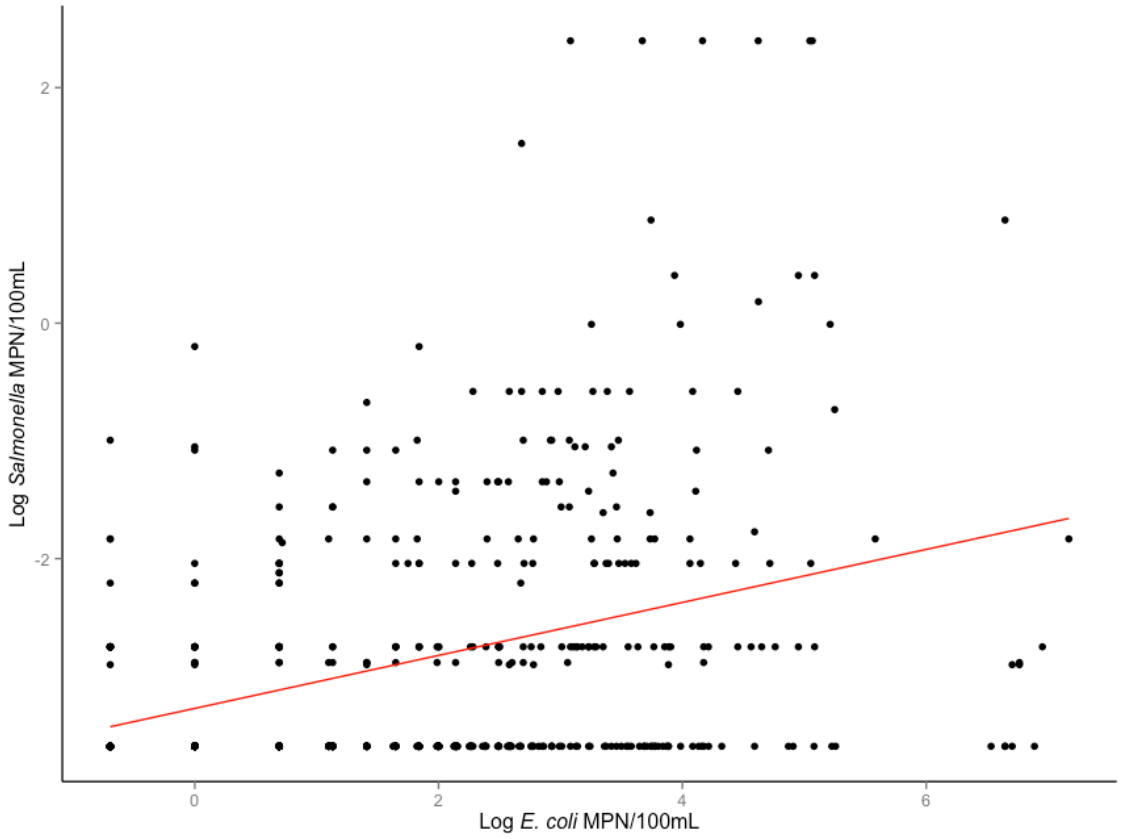
Supplementary Figure S1-1: Comparison of *Escherichia coli* concentrations by sampling strategy, stratified by the different sample types within each strategy. The bar plot compares the geometric mean of *E. coli* concentrations (left y-axis) for each sample type. "Comp" corresponds to the composite sample, created by combining aliquots of the three edge samples. "Intake" refers to the sample collected at the surface of the pond directly above the intake pump while "subs" samples were collected below the surface of the pond but above the intake pump. For each strategy (differentiated by color: white for strategy 1, grey for strategy 2), three edge samples (a, b, c) were collected. Composite, edge, intake, and subsurface samples are numbered according to their associated strategy (1 or 2). Error bars represent the standard error around the geometric mean. The scatter plot compares the proportion of positive samples (right y-axis) for each sample type.



Supplementary Figure S1-2: Seasonality of *Escherichia coli* concentrations (bar plot, left y-axis) and proportion of samples positive for *E. coli* (open circles, right y-axis) throughout the 19-month study (March 2012 to September 2013).



Supplementary Figure S1-3: Scatter plot showing correspondence of *Escherichia coli* and *Salmonella* spp. concentrations from study samples. In the final mixed effects model wherein log concentrations of *E. coli* and turbidity were predictors of log concentrations of *Salmonella*, the parameter estimate of log concentration of *E. coli* was 0.19 ($p < 0.001$).



Supplementary Table S1-1: Pairwise comparisons of *Salmonella* spp. concentrations at irrigation ponds.

Irrigation pond pair	Difference	Lower	Upper	*p* adj
LV-CC2*	-0.494	-0.924	-0.064	0.015
MD1-CC2	-0.097	-0.527	0.332	0.972
NP-CC2*	-0.742	-1.172	-0.312	0.000
SC-CC2	-0.262	-0.692	0.168	0.455
MD1-LV	0.396	-0.030	0.823	0.083
NP-LV	-0.248	-0.675	0.178	0.502
SC-LV	0.232	-0.195	0.658	0.571
NP-MD1*	-0.645	-1.071	-0.218	0.000
SC-MD1	-0.164	-0.591	0.262	0.829
SC-NP*	0.480	0.054	0.907	0.018

*Significant difference ($p < 0.05$)

Supplementary Table S1-2: Odds ratios (and 95% confidence intervals) for parameter estimates from logistic regression model for predicting *Salmonella* presence. Significant odds ratios are in bold.

	Odds Ratio	95% Confidence Interval
Log_{10} *E. coli* concentrations	**1.31**	**(1.10, 1.56)**
Presence of *E. coli* (Reference = absence of *E. coli*)	0.94	(0.37, 2.39)
Log_{10} Turbidity	1.11	(0.62, 2.01)
Total suspended solids	1.00	(0.99, 1.00)
Specific Conductivity	1.00	(0.67, 6.07)
Temperature	2.03	(0.89, 1.12)
pH	0.76	(0.51, 1.13)
ORP	1.00	(1.00, 1.01)

CHAPTER TWO

Salmonella Diversity and Distribution in Irrigation Ponds, Irrigation Systems, and Produce on Farms in Southern Georgia

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Abstract

To determine potential risks of irrigating commercial crops with surface water, this study investigated the presence and diversity of *Salmonella* in irrigation ponds (*n*=24), wells (*n*=5), irrigation distribution systems (*n*=65), and on produce (*n*=65) from four farms in southern Georgia. *Salmonella* was detected in water and produce samples, albeit in low concentrations (<1 MPN/100 ml water sample and <2 MPN/produce sample, respectively). We detected *Salmonella* in eight (33.3%) pond, 17 (26.2%) irrigation system, 1 (20%) well, and two (3%) produce samples. *Salmonella* isolates (*n*=38) were typed using serotyping, pulsed-field gel electrophoresis (PFGE), and whole genome sequencing. Ten serovars occurred in the samples, four of which (Newport, Javiana, Muenchen, and Saintpaul) are consistently isolated from clinical samples in Georgia. Seventeen discrete PFGE patterns were detected. No PFGE pattern co-occurred in all three sample types from the same farm (source waters, irrigation water, produce), but seven patterns were detected in ponds and irrigation systems from the same farm. On one farm, one pattern was detected in the irrigation system and on produce, but samples were collected two months apart. All but two PFGE patterns detected in this study were present in the Centers for Disease Control and Prevention's PulseNet Database. Whole genome sequencing uncovered additional diversity and revealed that study isolates clustered with human isolates across several continents. We conclude that our study in southern Georgia resulted in the isolation of low concentrations of clinically relevant *Salmonella* in surface water irrigation ponds, irrigation distribution systems, and on commercial produce.

Importance

Of bacterial foodborne pathogens, nontyphoidal *Salmonella* is responsible for the most illnesses and deaths in the United States (Scallan et al. 2011b). Several outbreaks have been attributed to fresh produce (Gelting et al. 2011; Greene et al. 2008). *Salmonella* is regularly detected in surface water in the southeast of the U.S., where surface water is used for irrigation on many farms (Antaki et al. 2016; Maurer et al. 2015). In this study, we assayed for *Salmonella* throughout the surface water-to-crop continuum, including in irrigation ponds, irrigation distribution systems, and on commercial produce. We detected various *Salmonella* serovars that are commonly isolated from human samples in this region. These results show that using contaminated surface water for fresh produce irrigation may pose a hazard to foodborne illness, although given the low concentrations, further research is needed to adequately characterize this risk. Our results demonstrate the temporal and spatial diversity of *Salmonella* in southern Georgia and the importance of future research in this region on environmental sources of human exposure to *Salmonella*.

Introduction

In the United States, foodborne pathogens cause more than 48 million illnesses annually (Scallan et al. 2011a; Scallan et al. 2011b). Despite increased surveillance of foodborne infections with the Foodborne Disease Active Surveillance Network (FoodNet), an extensive collaboration among the Centers for Disease Control and Prevention, the United States Department of Agriculture, the Food and Drug Administration, and state health departments, the incidence of foodborne illness from the most common pathogens has largely remained the same (Crim et al. 2015). Nearly half of foodborne illnesses can be attributed to produce consumption (Painter et al. 2013) and fresh produce has been

implicated in numerous outbreaks in the United States (Gelting et al. 2011; Greene et al. 2008; Hanning et al. 2009; Uyttendaele et al. 2015). Thus, produce safety is critical to reducing the burden of foodborne disease in the United States. To address the contribution of produce contamination in the promotion of food safety, the Food and Drug Administration recently issued science-based standards for the safe production, harvesting, and processing of fruits and vegetables through the Produce Safety Rule of the Food Safety Modernization Act (Food and Drug Administration 2015).

Fresh produce can be contaminated with enteric pathogens along numerous pathways from farm to fork (Franz and van Bruggen 2008; Mandrell 2009). Many studies have investigated the postharvest potential for contamination of crops, such as during packaging and processing. Fewer studies have examined the factors driving contamination risk at the preharvest stage of production, even though preharvest contamination has been implicated in several outbreaks (Gelting et al. 2011; Greene et al. 2008). Moreover, when transport, packing, and storage conditions are amenable to bacterial growth, pathogen concentrations could potentially increase exponentially en route from farm to the point of consumption (Tomás-Callejas et al. 2011). Additionally, preharvest contamination could potentially introduce pathogenic bacteria to packing facilities and lead to cross-contamination (Murray et al. 2017). This complicates efforts to prevent foodborne illnesses as well as traceback efforts (already made difficult by the complexities of the food supply chain) following potential outbreaks to identify the root cause of contamination.

Fresh produce can become contaminated with enteric pathogens in the field through irrigation with contaminated water, direct contact with contaminated soil, and splash during

irrigation or rainfall events that transfer soil contaminants to edible portions of crops (Cevallos-Cevallos et al. 2012; Franz and van Bruggen 2008; Kisluk and Yaron 2012; Liu et al. 2013). Microbial contaminants in soil on farms may be introduced through the application of biological soil amendments of animal origin and the intrusion of domesticated animals and wildlife (Jacobsen and Bech 2012; Park et al. 2012). In addition to coming into direct contact with crops, contaminants in soil can also be transported into surface water and groundwater through precipitation runoff and leaching, respectively (Mawdsley et al. 1995).

The survival and transport of enteric pathogens in soil and water is particularly troubling in areas where pathogens are also present in the produce production environment. In southern Georgia, *Salmonella* has been regularly detected in surface water ponds used for irrigation (Gu et al. 2013; Haley et al. 2009; Harris et al. 2018; Li et al. 2014) but concentrations were low, often less than 0.40 MPN/1 L (Antaki et al. 2016; Luo et al. 2015). To examine the risk of produce contamination on farms that use surface water for irrigation, and in particular to follow up on a study by Antaki et al. (2016), which detected *Salmonella* in irrigation ponds and in irrigation systems but did not sample produce, we carried out a study to determine the extent to which *Salmonella* may be transported from irrigation ponds, through irrigation systems, and onto produce.

Materials and Methods

Study Sites

Samples were collected from four mixed-use produce farms in the Little River watershed of the Coastal Plain of southern Georgia. These farms use surface water irrigation ponds located adjacent to the fields. Per our agreement with the growers, we have not disclosed the

identity or location of our sites. The four sites will hereinafter be referred to as HP, LV, SC, and WL. At Farm SC, two pumping stations withdrew water from the same pond and depending on the pumping station used, downstream water and produce samples are referred to as SC1 and SC2. Farms LV and SC have previously been analyzed for *Salmonella* by Li et al. (2014) and Farms LV and HP were studied by Antaki et al (2016).

Sample Collection

Water samples: From May to November 2014, we collected 94 water samples from irrigation systems (three center pivot systems with overhead sprinklers, two solid set sprinkler systems, three drip irrigation systems) and irrigation water sources (four irrigation ponds and one deep groundwater well). For each crop harvest, water samples were collected during three irrigation events that occurred 2-45 days before the start of the commercial produce harvest. These three sampling events occurred at regularly spaced intervals in the 2-45 days before harvest. At each event, samples were collected from the irrigation water source. When the water source was a pond, samples were collected near the intake of the pump used to divert water from the pond to the irrigation system. A peristaltic pump with sterile Tygon® tubing (Saint-Gobain, Courbevoie, France) was used to collect pond water at a depth of approximately 1 m while the irrigation pump was in use. Samples were also collected from a water valve/spigot installed downstream from the irrigation pump but upstream of the pump's filtration system. Filtration systems varied by location but were generally sequential sand-bed filters. For fields irrigated with well water, 2 L samples were taken from the well spigot. Drip irrigation samples were collected from the start and end of drip lines and were 2 L composites of three randomly selected drip line samples. Pivot and solid set sprinkler samples were 2 L composites collected from six randomly selected sprinklers with the same

irrigation water source. Sprinkler samples were collected in sterile 2 L plastic containers held just below the sprinkler for pivots and facing the sprinkler for solid set systems. Samples were stored on ice and analyzed in the laboratory within 24 hours of collection.

Produce samples: Produce sampling took place two to three days following the third and final water sampling event. Produce samples were collected using sterile 1.63 L Whirl-Pak® bags (Nasco, Fort Atkinson, WI). The number of produce units for each sample bag varied across crop types. Prior to the commercial harvests, to determine the number of produce units to collect for each sample, 2 L of water were added to a Whirl-Pak® bag and produce units were individually added to the bag to determine the maximum number of units that could be fully submerged in the bag. This process allowed us to maximize the number of produce units per sample while making sure that each sample could be analyzed in the laboratory per our *Salmonella* enumeration protocol. *Salmonella* enumeration in the laboratory for produce samples (to be described in greater detail in the following section) involved washing produce with 2 L of buffered peptone water and enumerating *Salmonella* in the wash. This 2 L wash volume was chosen to be consistent with the volume of water samples. Five produce samples were collected for each harvest. Samples of broccoli (5 crowns/sample), mustard greens (30 leaves/sample), cucumbers (8 units/sample), bell peppers (6 units/sample), watermelon (1 unit/sample), cantaloupe (2 units/sample), zucchini (8 units/sample), and squash (6 units/sample) were collected between January 2014 and November 2014. Produce samples were stored on ice and analyzed in the laboratory within 24 hours of collection. A complete list of samples collected by date and number of produce units per sample can be found in Supplementary Table S2-1.

Salmonella Enumeration

The full laboratory workflow for sample analysis can be found in Supplementary Figure S2-1. Water samples were analyzed for *Salmonella* using a cross-streaking method described by Luo et al. (Luo et al. 2015) This most probable number (MPN) method (limit of detection: 0.0548 MPN/100 ml) used three dilutions (in triplicate) of 2x lactose pre-enrichment broth: 500 ml, 100 ml, and 10 ml (Becton, Dickinson and Company (BD) Difco™, Franklin Lakes, NJ). Equal volumes of the wash were added to each dilution (9 total) and samples were incubated at 37°C for 24 hours. One ml of each pre-enriched dilution was further enriched with 9 ml of *Salmonella*-selective tetrathionate broth with iodine (Remel, Lenexa, KS). After a 24-hour incubation at 37°C, 10 µl of each sample was plated onto Xylose-Lysine-Tergitol 4 (XLT-4) agar (Becton, Dickinson and Company (BD) Difco™, Franklin Lakes, NJ), a *Salmonella*-selective agar, and plates were incubated at 37°C for 24 hours. Presumptive positives were then streaked onto CHROMagar™ *Salmonella* Plus (CHROMagar Microbiology, Paris, France), another selective media for the isolation of *Salmonella*. If only one replicate per sample was a presumptive positive, three isolates from this replicate were randomly selected from CHROMagar™ plates for downstream analyses. If more than one replicate per sample was a presumptive positive, six colonies were randomly selected to represent the sample. For samples with fewer than six colonies, all presumptive positive isolates were selected.

Produce samples were analyzed similarly, with the addition of an initial produce wash step, and a storage step. Following collection in Whirl-Pak® bags, produce samples were brought to the laboratory, where each of the five samples was washed with 2 L of buffered peptone water (Remel, Lenexa, KS); samples were shaken for 30 seconds, massaged for 60 seconds,

and shaken for another 30 seconds. Produce rinsates were pre-enriched with lactose broth as described for the water samples (500 ml, 100 ml, and 10 ml in triplicate). After this initial incubation however, 10 ml samples (15% glycerol) were stored at -80°C for 90 days. This was done in accordance with our agreement with the growers in this region and has been described elsewhere (Benjamin et al. 2013; Gorski et al. 2011). At the end of the 90-day period, samples were thawed and resuscitated following a protocol optimized in our laboratory (Pennington 2014). Samples were thawed at 24°C for 2 hours and incubated at 37°C for 4 hours. One-milliliter aliquots of the resuscitated, pre-enriched samples were added to 9 ml of tetrathionate broth with iodine and samples were subsequently analyzed as previously described for the water samples.

PCR was used to confirm suspected *Salmonella* isolates using primers targeting the *invA* gene (Chiu and Ou 1996). To extract DNA from samples presumed positive by XLT-4 and/or CHROMagar *Salmonella* Plus, each *Salmonella* colony was placed in 200 µl of molecular grade water and boiled at 100°C for 10 minutes to lyse the cells. After 10 minutes, samples were cooled on ice and then centrifuged at 15,000 g for 5 minutes. The supernatant (containing the extracted DNA) was stored at -20°C. DNA extractions were used as template for 25 µl PCR reactions containing 10 µl of 2x Promega Master Mix (Promega, Madison, WI), 5 µl each of the forward and reverse primers at 10 µM concentration, *invA*-1 (5'-ACAGTGCTCGTTTACGACCTGAAT-3') and *invA*-2 (5'-AGACGACTGGTACTGATCGATAAT-3'), and 5 µl of template DNA. Samples were subject to an initial denaturation step at 95°C for 1 minute and then cycled 30 times at 95°C for 1 minute to denature, 56°C for 30 seconds to anneal, and 72°C for 1 minute to extend. Cycles were followed by a final extension at 72° C for 5 minutes. DNA extracts from

Salmonella isolates from environmental samples in the study region were used as positive controls for PCR reactions. PCR products were separated using a 2% agarose gel and bands were visualized using UV transillumination with a UV gel imager (Bio-Rad, Hercules, CA). Confirmed *Salmonella* isolates were preserved in 15% glycerol and stored at -80°C until further analysis consisting of serotyping, pulsed-field gel electrophoresis (PFGE), and whole genome sequencing.

Selection of Isolates for Subtyping Analyses

Isolates from the same sample with identical PFGE patterns were deemed clones and only one of these isolates was used in serotyping and whole genome sequencing.

Serotyping Analysis

To serotype isolates, samples were sent to the California Animal Health and Food Safety Laboratory (CAHFS) in San Bernardino, California (n=43). Samples that could not be serotyped by CAHFS were sent to the National Veterinary Services Laboratory in Ames, Iowa for further confirmation.

PFGE Analysis

Salmonella isolates were fingerprinted with PFGE using *XbaI* digest. Samples were analyzed using a protocol standardized by the Centers for Disease Control and Prevention (CDC) for PulseNet data (Antaki et al. 2016). *Salmonella* Braenderup ATCC BAA664 was used as a molecular size standard for normalization. Isolates with indistinguishable gel patterns were digested with a second enzyme, *BlnI*, and 50 μM thiourea (Sigma-Aldrich, St. Louis, MO) was added to the 0.5 Tris-borate-EDTA (TBE) buffer.

PFGE gel images were analyzed using the BioNumerics software 7.1 (Applied Maths, Kortrijk, Belgium). Similarity analysis was performed using Dice coefficients at an optimization of 1.5% and a band position tolerance of 1.5%. Similar isolates were clustered using the unweighted-pair group method with arithmetic mean (UPGMA).

PulseNet

In order to assess the human disease relevance of the pulsotypes detected with PFGE, gel images were used to query the PulseNet database operated by the CDC. Isolates were compared to PFGE patterns in the database from January 1998 to December 2016. Images from 38 isolates digested with *XbaI* and the additional eight digested with *BlnI* were compared to the entries in the PulseNet database.

Whole Genome Sequencing

Salmonella isolates were sent to the Food and Drug Association's Center for Food Safety and Applied Nutrition (FDA-CFSAN) Molecular Methods and Subtyping Branch in College Park, MD for whole genome sequencing.

All isolates were extracted using the gram-negative DNA extraction protocols described in the DNeasy Blood & Tissue Kit User Manual (Qiagen, Germantown, MD, USA). The resulting DNA samples were then constructed into DNA libraries using either the Nextera XT DNA Library Preparation Kit (Illumina®, San Diego, CA, USA), using an initial DNA input of 0.2 ng/μl and performed according to the manufacturer's instructions.

All sequencing described in this study was performed on Illumina® MiSeq or NextSeq desktop sequencers. Sequencing performed on the NextSeq was done using the 2x150 cycle (High) kit (Illumina®, San Diego, CA, USA). Sequencing on the MiSeq platform was accomplished using the 2x300 cycles (V3) kit or the 2x250 cycles (V2) kit.

Sequencing records (IIlumina MiSeq n= 120, Illumina NextSeq = 186) that passed our quality control filters: average sequencing depth of coverage higher than 20X and average quality score for read 1 & 2 at 27 or higher, have been deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under two different *Salmonella* Bioprojects: PRJNA308180 (MiSeq data) and PRJNA186035 (NextSeq data).

Raw data was subjected to genome assembly using Spades version 3.8. Reads shorter than 500 bp were removed and genome annotation was performed on filtered assemblies with NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

A whole genome phylogeny was generated from the genome alignments of *Salmonella* isolates collected from the environmental sources used in this study. The genome sequences were aligned using Mugsy x86-64-v1r2.3.1 (Angiuoli and Salzberg 2011) and the concatenated regions from each genome were used to construct a maximum-likelihood phylogeny using RAxML v7.2.8 (Stamatakis 2006). The phylogeny was generated with 100 bootstrap replicates and visualized using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

We compared the SNP differences among the sequences within a phylogenetic cluster to assess the sequence diversity of our samples (Deng et al. 2003). A de novo SNP analysis was conducted using the NUCmer application from MUMmer 3.23.

Sequences were deposited into the GenomeTrakr database at NCBI. NCBI maintains a real-time phylogenetic tree comparing all of the *Salmonella enterica* sequences uploaded to its database (https://www.ncbi.nlm.nih.gov/pathogens). We assessed clustering and phylogenetic similarities between our isolates and other *S. enterica* isolates across the country in the database as of February 2, 2018.

Serovar Predictions

In addition to the serovar determinations made by serology-based serotyping, we examined the serovars for each isolate as determined by their PFGE patterns and whole genome sequences (WGS). In addition to PFGE patterns, PulseNet entries contain serotyping data for each isolate. For each isolate, we compared its serovar to the predominant serovar within the PFGE pattern cluster in PulseNet. We also examined the serovar predictions from the WGS data using the *Salmonella* in silico Typing Resource (SISTR), a platform which generates serovar predictions from isolate genomes in two ways: using the antigenic formula (O serogroup, H1, and H2 antigen gene sequences) and the "phylogenetic context," which uses the predominant serovar within cgMLST clusters (Yoshida et al. 2016).

Salmonella Diversity Analysis

To estimate *Salmonella* type richness in our study and determine whether further sampling would uncover more types of *Salmonella* in our study region, species accumulation curves

were constructed using serovar and PFGE pattern data. We estimated species accumulation curves using the vegan package in R (R Core Team 2015).

Statistical Analyses

For statistical analyses, samples in which *Salmonella* could not be detected were set to half of the lower limit of detection (lower limit = 0.0548 MPN/100 ml). For descriptive statistics, we calculated geometric means of *Salmonella* concentrations by sample type.

Results

Salmonella Enumeration

Twenty-seven of the 94 total water samples (29%) tested positive for *Salmonella*. *Salmonella* concentrations were low—they ranged from 0.055 MPN/100 ml to 0.99 MPN/100 ml. All sample types tested positive for *Salmonella* at least once, except for the solid set sprinkler. The full summary of sample results is described in Table 2-1.

Of the 65 produce samples collected, two (3%) tested positive for *Salmonella*. One of these positive samples was a cantaloupe sample collected from Farm HP in June 2014. The *Salmonella* concentration for this sample was 1.1 MPN per two cantaloupes. Cantaloupe crops were grown on narrow bed plastic and irrigated with pond water via a center pivot sprinkler system. Pond and sprinkler samples collected two days before the produce harvest were also positive for *Salmonella*. The *Salmonella* concentration in the pond sample was 0.37 MPN/100 ml and the concentration in the pivot sample was 0.99 MPN/100 ml. These concentrations were the highest detected levels on any sample throughout this study. The other positive produce sample was from a cucumber collected from Farm SC (SC1 pumping

station) in July 2014. *Salmonella* was detected at a concentration of 1.28 MPN per eight cucumbers. Cucumber crops were grown on raised beds covered with plastic mulch and irrigated with pond water via a drip tape irrigation system. The drip tape was located under the plastic mulch. *Salmonella* was not recovered from any of the pond and drip irrigation samples collected prior to the cucumber harvest.

Selection of Isolates for Subtyping Analyses

A total of 143 confirmed *Salmonella* isolates (from the 27 positive water samples and 2 positive produce samples) were analyzed using PFGE. Following clone removal, 43 isolates remained: 40 from water and 3 from produce. These 43 isolates were sent for serotyping and whole genome sequencing. However, following serotyping and whole genome sequencing, another five water isolates were deemed clones. Analyses of relatedness and diversity used the final set of 38 distinct isolates.

Serovar Analysis

Eleven serovars were identified from the 43 *Salmonella* isolates deemed distinct via PFGE analysis (Table 2-2). The three most common serovars were *Salmonella* Saintpaul (10 isolates, 26%), Muenchen (9 isolates, 24%), and Newport (6 isolates, 16%). Saintpaul, Muenchen, and Newport were prevalent throughout the study occurring at multiple farms and were detected in irrigation ponds and their associated irrigation systems. The isolates from the well spigot and drip irrigation system sourced by this well were serotyped as *Salmonella* Newport. Of the 38 distinct isolates, some serovars (Javiana, Rubislaw, III_60:r:e,n,x,z15, III_16:z10:e,n,x,z15) were only represented by one isolate. Other serovars (Hartford, Muenchen) were only detected at one farm but were detected in both irrigation ponds and distribution systems.

The serovars detected in the produce samples were not detected in any of the water samples. The Rough "O" *Salmonella* serovar was isolated from both the cucumber and the cantaloupe samples. In addition to the Rough "O" serovar, *Salmonella* Bardo was isolated from the cucumber sample.

PFGE Analysis

Seventeen discrete fingerprint patterns emerged from PFGE analysis of 43 isolates (Figure 2-1). More than half of the PFGE patterns (9 of 17) were represented by only one isolate (Patterns 2, 3, 6, 7, 9, 11-13, 17; hereafter referred to as singletons); the remaining eight PFGE patterns were detected across sample types and/or farms.

Some of the PFGE patterns were detected at multiple points along the pathway from water source to produce within a given farm. At Farm HP and Farm SC, there were three PFGE patterns present in the pond that were also present in the irrigation systems that withdrew water from these ponds. At Farm SC, PFGE Pattern 1 was detected in the drip irrigation system of SC1 in May and was subsequently detected on one of the two isolates cultured from cucumbers (also from SC1) in July. Pattern 1 was also detected on the isolate cultured from cantaloupes from Farm HP in June. Pattern 1 appears common—it was detected at all 4 farms over the course of the study. In contrast, the pattern from the other isolate cultured from cucumbers did not match any of the other patterns in our data. The diversity of PFGE patterns by farm and sample type (source water, irrigation system, produce) over the study period is shown in Figure 2-2.

PulseNet Query

Of the 17 PFGE patterns, 15 patterns matched entries in CDC's PulseNet database (Table 2-3). However, isolates from this study were not linked to any specific outbreaks during the sample period. Two patterns (Patterns 7 and 9) did not match any pattern in the database; these were both also singletons in our PFGE analysis. Pattern 7 was isolated from a drip irrigation system from Farm SC and Pattern 9 was isolated from a pivot sample from Farm HP. Serotype analysis indicated that these isolates were both *Salmonella* Muenchen.

Whole-Genome Sequencing

The whole-genome phylogeny clusters were similar to the clustering of isolates in the PFGE pattern dendrogram. However, the sequences provide finer resolution to distinguish individual isolates from one another. For example, some of our positive isolates were indistinguishable by PFGE from our positive controls, which were all Newport strains that had previously been isolated from our study region. However, single nucleotide polymorphism (SNP) analysis indicated substantial differences between the positive controls and these isolates. In addition, SNP analysis indicated a great degree of diversity among the other sequences of this cluster. In one instance, two isolates of the same serovar and PFGE pattern had SNP differences of 631 base pairs. Figure 2-3 shows the WGS dendrogram for a portion of isolates (including the produce isolates) with highly related sequences (Supplementary Table S2-2). Sequences of samples from this study have been uploaded to the NCBI Sequence Read Archive. A full list of the accession numbers can be found in Supplementary Table S2-3.

Our comparison of *Salmonella enterica* sequences from this study to others in the NCBI database indicated that our isolates were part of 11 SNP clusters (Table 2-4). The three produce isolates belong to one cluster in this real-time tree that also contained clinical samples from four states in the U.S., Canada, and the United Kingdom, and environmental samples from 12 states in the U.S. and Peru. The largest cluster containing isolates from our study included 351 clinical samples from Canada, Taiwan, the United Kingdom and the U.S. and environmental samples from Central America, South America, Europe, North Africa, East Asia, South Asia, the Middle East, and North America. A dendrogram of the SNP cluster containing the produce isolates can be found in Supplementary Figure S2-2. Dendrograms of the other ten SNP clusters can be found on the NCBI website using the nucleotide sequence accession numbers for our samples.

Serovar Predictions

Using the predominant serovar within PFGE pattern clusters in PulseNet led to three instances of serovar mismatch (as compared to our serotyping analysis). For three isolates, the predicted serovar was Newport when our serotyping analysis indicated that the serovars were Rough "O" and Bardo. However, Rough "O" and Bardo are considered to be variants of Newport. Predicting serovars using the antigenic formula method of SISTR also led to the same three serovar mismatches. All serovar mismatches are indicated in Table 2-3.

Diversity of Salmonella *Types Detected*

Of the 43 isolates analyzed with serotyping and PFGE, 11 serovars and 17 discrete fingerprint patterns were detected. At Farms HP and SC, there were multiple PFGE patterns detected even on the same day of sampling (Figure 2-2). At Farm HP, seven PFGE patterns

were detected from samples collected on one day of the study. *Salmonella* concentrations in our study samples were low and yet, numerous PFGE patterns were present. On the day of recovering seven PFGE patterns from HP, the concentrations in the pond and pivot samples were 0.99 and 0.37 MPN/100 ml, respectively.

We also found great within-sample diversity—multiple serovars and PFGE patterns were recovered from the same sample. Up to three different serovars and five different PFGE patterns could be detected in one sample. The PFGE subtype and serovar accumulation curves were developed to estimate species richness in our study region (Figure 2-4). These curves indicate that our sampling efforts have captured the diversity of serovars in our study region but further sampling has the potential to reveal more PFGE pattern diversity. Together, these results indicate that a high level of *Salmonella* diversity may be present in our study region.

Furthermore, whole genome sequencing and SNP comparisons of these sequences showed an even higher level of genetic diversity among isolates of the same PFGE pattern (Figure 2-3). Given the greater discriminatory capacity of WGS, we would expect the WGS accumulation curve to be steeper than the curves for the serovars and PFGE patterns and that increased sampling efforts would allow us to uncover even more *Salmonella* diversity in our study region.

Discussion

The presence of *Salmonella* in the natural environment of southern Georgia is well-documented (Antaki et al. 2016; Haley et al. 2009; Luo et al. 2015; Maurer et al. 2015) and in

prior studies, our research group has also been able to detect *Salmonella* in irrigation distribution systems and the surface water irrigation ponds that feed them (Antaki et al. 2016). In these studies, we determined that *Salmonella* could be regularly detected at low concentrations in surface water and irrigation water distribution systems on farms in southern Georgia between May and October. Here, we extend these results to provide the first evidence in this region that *Salmonella* contamination exists in source waters, irrigation systems and on commercially harvested crops.

We recovered *Salmonella* from surface water, well water, irrigation systems, and on produce. Produce on these farms may have been contaminated by irrigation with surface water, which has been cited as a potential source of crop contamination (Islam et al. 2004; Oliveira et al. 2012; Weller et al. 2015b). This is further supported by our detection of *Salmonella* in surface water irrigation ponds and in irrigation systems of study farms. The surface water irrigation ponds in our study were accessible to wild animals, such as birds and reptiles, which are known reservoirs of *Salmonella* in this region and thus, were susceptible to contamination (Hudson et al. 2000; Srikantiah et al. 2004).

We found *Salmonella* with the same PFGE patterns in surface water irrigation ponds and the irrigation systems that sourced water from these ponds. However, these patterns were not always found sequentially, with the detection of the pattern in source water before or concurrent with detection in irrigation systems. We also found isolates with the same PFGE pattern in irrigation distribution lines and on the crops irrigated by these systems (Pattern 1). However, we did not find isolates of the same PFGE pattern in source water, irrigation systems, and on crops from the same fields. This—and the sequence of pattern detection in

ponds and irrigation systems—may indicate contamination sources other than irrigation water, such as wildlife intrusion and contact with pathogens in soil or it may be due to undersampling, as indicated by the species richness curve for PFGE patterns (Figure 2-4). In a study by Jokinen et al. (2010), many of the *Salmonella* serovars and phagetypes detected in wildlife from the Salmon River watershed were not the same as the serovars and phagetypes detected in surface water in the watershed. In a similar vein, while 14 of 37 serovars detected by Maurer et al. (2015) in Georgia were present in both wildlife and surface water, the remaining serovars were either only detected in wildlife or in surface water. It is possible that surface water bodies in the present study have numerous sources of contamination and that wildlife can contribute to the contamination of surface water but also to the direct contamination of produce and soil. In our correspondence with growers on these farms, we discovered that only inorganic fertilizers and pesticides were applied, ruling out the possibility of contamination through the application of untreated biological soil amendments of animal origin, such as poultry litter. Similarly, *Salmonella* serovars commonly associated with food animal production were rarely detected by Maurer et al. (2015) in surface water, even in areas where poultry production was prevalent.

Salmonella Diversity

The PFGE, serotyping, and WGS results suggest high spatial and temporal diversity of *Salmonella* on produce farms in this region. The study revealed numerous PFGE patterns in ponds and irrigation systems even in samples that had low concentrations of *Salmonella*. Certain subtypes are prevalent and persistent throughout the study region, such as Patterns 1, 14, and 15, which were found across different farms and sample collection dates. Some of the PFGE patterns were only represented at one farm, while others were detected at

multiple farms (e.g. patterns 1, 14, 15). Similarly, some patterns occurred only once throughout the year while others commonly reoccurred (e.g. patterns 1, 5, 10, 15, 16). The reoccurrence of some PFGE patterns may indicate a constant source of contamination, as observed in one study where non-migratory birds that were present throughout the year were potential sources of *Salmonella* contamination in urban streams (Thomas et al. 2013). In contrast, sporadically occurring PFGE patterns may indicate intermittent contamination events from a source. For example, PFGE pattern 4 was only detected during one sampling event on one farm throughout the study. In a study of *Salmonella* contamination of drinking water, Refsum et al. (2002) detected the same PFGE pattern from gulls, a gull feather sample in the drinking water supply, and a salmonellosis outbreak in the same area of Norway. Further surveillance efforts were unable to find the same PFGE pattern in other gull samples suggesting the possibility of an isolated drinking water contamination event. These irregular occurrences of some *Salmonella* subtypes in the environment may also be due to undersampling; greater sampling efforts might reveal that these subtypes are frequently present in the environment.

Our results are consistent with prior studies that detected a wide variety of PFGE patterns in irrigation pond water and irrigation systems in this region and provide further evidence that water bodies in this region are rich with *Salmonella* diversity (Antaki et al. 2016; Li et al. 2014; Maurer et al. 2015). The most common PFGE patterns detected in this study were not the same patterns found by Li et al. (2014), suggesting that there is regular turnover of the dominant *Salmonella* subtypes. Maurer et al. (2015) detected 204 unique *Salmonella* PFGE patterns in Georgia but 82% of these patterns were only detected once from 2005-2011.

The genetic diversity detected in this study suggest that *Salmonella* contamination in this region is likely not a result of an isolated instance of point source contamination; instead, *Salmonella* introduction onto these produce farms may occur from the contribution of multiple diffuse environmental sources. Other studies have found that the diversity of *Salmonella* subtypes in surface water may have occurred from various sources in the environment, including sewage and myriad wild and domestic animals (Gomba et al. 2016; Gorski et al. 2011; Jokinen et al. 2010; Maurer et al. 2015; Topalcengiz et al. 2017; Walters et al. 2013). This is further corroborated by our estimates of species richness which indicate that even though we detected a high level of genetic diversity in our study isolates, there may be an even higher level present on these produce farms that we were unable to fully capture. Through a similar use of the PFGE pattern accumulation curve, Walters et al. (2013) also determined that greater sampling efforts in Central California had the potential to detect even more *Salmonella* subtypes. As previously mentioned, Maurer et al. (2015) detected 204 *Salmonella* PFGE patterns from 2005-2011 in the state of Georgia. The present study detected fewer serovars and PFGE patterns but only spanned one year in southern Georgia; thus, continued surveillance may uncover even more subtype diversity. WGS results confirm the genetic diversity of the *Salmonella* pangenome in this region, evident within both a single sample and even within a single PFGE pattern. Similarly, in an analysis of 52 *Salmonella* isolates of the Enteritidis serovar by Deng et al. (2015), eight PFGE patterns were detected. In contrast, 34 subtypes were detected using WGS. Thus, WGS has the discriminatory power to distinguish between isolates that have the same PFGE pattern. This suggests that WGS can be used in future studies to more effectively determine the sources of *Salmonella* contamination. These findings on *Salmonella* diversity on commercial farms emphasize the

importance of characterizing the full range of environmental reservoirs of *Salmonella* in the produce production environments of southern Georgia.

Clinical Relevance

The results of the PulseNet query indicate that many of the patterns and serovars detected during this study have previously been implicated in cases of human salmonellosis (Table 2-3). Pattern 1 was the 10th most common pattern entry in the PulseNet database in 2014, the year of our study. While the 14 other PFGE patterns were not as prevalent in the database. Our results suggest that the *Salmonella* subtypes in our study are clinically relevant, which is consistent with findings from prior studies that have also examined the clinical relevance of *Salmonella* subtypes in this region (Li et al. 2014; Maurer et al. 2015).

Similarly, four of the 10 serovars that we detected (*Salmonella* Javiana, Newport, Muenchen, and Saintpaul) are consistently some of the most common serovars associated with foodborne illness. The most common serovars in this study (Muenchen, Saintpaul, and Newport) are also frequently isolated in clinical cases in Georgia (Georgia Department of Public Health 2015) and in the United States (Centers for Disease Control and Prevention 2017b). In 2013, *Salmonella* Newport and Javiana were responsible for over 30% of the cases in Georgia (Georgia Department of Public Health 2015). These two serovars were also detected in the environmental samples from this study.

The whole genome sequences of our isolates provide finer resolution in our examination of the clinical relevance of our isolates. Of the 11 real-time phylogenetic clusters to which our samples belonged, six clusters contained sequences from clinical samples.

Southern Georgia consistently has some of the highest salmonellosis incidence rates in the country—in 2015, there were 20.69 cases per 100,000 people in Georgia, compared to 15.74 cases per 100,000 in the country overall (Centers for Disease Control and Prevention 2017b). Many of these salmonellosis cases are not outbreak-related. Given the regular detection of *Salmonella* in surface water in this region, environmental exposure may be an underestimated cause of disease in Georgia. However, it is important to note that, while *Salmonella* was detected in all sample types tested and we found isolates that matched instances of human cases in reference databases, only a small proportion of produce items sampled tested positive. This low proportion of positive produce samples might be due to the limits of detection in this study; *Salmonella* concentrations on positive samples were low and thus, for some produce samples, the levels of contamination may have been below the detectable threshold. This low proportion might also be explained by the low concentrations of *Salmonella* we detected in irrigation ponds and irrigation water. In a study by Kisluk and Yaron (2012), *Salmonella* could not be detected on parsley irrigated with 1.5 log CFU/ml of *Salmonella*. The highest concentration of *Salmonella* detected in irrigation water in the present study was 0.99 MPN/100ml and thus, it is possible that *Salmonella* concentrations on crops were too low to be detected in our produce samples. Moreover, the salmonellae that might have been applied to crops may have experienced die-off in the 2-3 days between irrigation and our time of produce sampling, given the die-off that can occur on plant surfaces within 48 hours of contamination (Kisluk and Yaron 2012; Weller et al. 2015b). However, another explanation for the low prevalence is that *Salmonella* in irrigation water may have been internalized by the plants (Erickson et al. 2012; Franz et al. 2007) and thereby not captured by our assays that only tested plant surfaces.

By analyzing isolates with different methods, this study was able to compare serovar predictions using PFGE fingerprint patterns and WGS with traditional serotyping (Table 2). Prior studies have demonstrated the capacity of using PFGE patterns and WGS for serovar prediction (Gaul et al. 2007; Yachison et al. 2017; Zou et al. 2012), which is particularly useful with the growing accessibility WGS. For most isolates in our collection, the three prediction methods agreed on serovar classification. In one of the mismatches, the two serovars predicted were actually the same—the Rough "O" serovar is considered to be a Newport variant. *Salmonella* isolates are considered "rough" when they do not express their O antigens. It has been posited that *Salmonella* spp. may not express their O antigens when subject to environmental stressors, such as limitations in nutrients (Gerstel and Römling 2001). Future studies should investigate whether morphological changes in *Salmonella* occur on plant surfaces.

Study Limitations

This study was limited by the feasibility of representative sampling at these large farms. For the water samples, greater prevalence and diversity of *Salmonella* may have been detected with the use of large volume ultrafiltration methods (Kahler et al. 2015; Mull and Hill 2009, 2012).

For the commonly occurring PFGE patterns, some PFGE patterns may be dominant in this study system, but it is also possible that some of the patterns were commonly found due to culture bias. The numerous culture-based steps in the isolation of *Salmonella* may have selected for certain *Salmonella* isolates over others. This bias may have been exacerbated during the produce sample resuscitation efforts. The protocol was optimized to ensure the

accurate recovery of *Salmonella* concentrations but not necessarily diversity. In future studies, as indicated by Strawn et al. (2014), multiple isolation schemes should be used to optimize *Salmonella* recovery for improved analysis of extant *Salmonella* diversity in a given region (Strawn et al. 2014).

Conclusions & Future Research Directions

Increased focus on studying *Salmonella* in Georgia is necessary to better understand this pathogen's survival and transport in the natural environment. These efforts will help identify leverage points to inform growers about produce safety risks and mitigate exposure to *Salmonella*. This study has indicated that surface water contamination is a potential risk for irrigation water quality and produce contamination. While this points to the role of irrigation water quality in produce safety, it is also possible that crops were contaminated through other routes. Some of the PFGE patterns on produce were not detected in any of the water samples and only one PFGE pattern was detected on both produce and water samples. None of the PFGE patterns was detected in surface water, irrigation systems, and on produce. As previously mentioned, this may be a result of undersampling. However, it is important for future studies to look into other modes of contamination on these produce farms. Of note, cantaloupe and cucumber, the two produce types that were positive, mature close to the soil. Crops may have been contaminated via direct contact with pathogens on soil sediment or splash transfer during rainfall or irrigation events. Future work should pursue the potential for preharvest contamination of crops due to interactions with soil.

Additionally, future work should model die-off of *Salmonella* on produce in areas where growers irrigate with surface water contaminated with *Salmonella*. Weller et al. (2015b) found

that waiting three days after irrigation to harvest was associated with reduced risk of *Listeria* on produce. A development of a science-based waiting period for *Salmonella* will help provide growers with an appropriate intervention to minimize the presence of *Salmonella* at the time of harvest. In addition to the implementation of a harvest interval, growers often chlorinate surface water used for irrigation of crops and processing facilities chlorinate water used to wash produce (Gil et al. 2015; Murray et al. 2017; Park and Kang 2015; Suslow 2003; Suslow 2005). There have been several studies that evaluate the impacts of various produce safety interventions and model the risk of foodborne illness from fresh produce consumption through quantitative microbial risk assessments (QMRA) of the entire supply chain, from preharvest conditions in the field to the point of consumption (Danyluk and Schaffner 2011; Maffei et al. 2017). Future QMRA work can adapt these aforementioned models to assess the risks of *Salmonella* on produce irrigated with surface water by incorporating our findings of low concentrations and prevalence of *Salmonella* on produce.

Future risk assessments should also consider the unintended health risks of produce safety interventions. One consequence of chlorination may be a shift in the bacterial community present in the agricultural environment that results in the increased relative abundance of antibiotic resistant genes (Jia et al. 2015; Xi et al. 2009). Another consequence may be chronic exposure to chlorination byproducts through produce consumption (Suslow 2003; Trinetta et al. 2011). The previously described QMRA models examined varying levels of chlorine used to minimize the risk of *Salmonella* contamination and found increased risk of produce contamination when chlorine levels were low. These results show the importance of adequate chlorination of water that comes into contact with edible portions of crops. However, the health risks of exposure to chlorination byproducts are not well understood

(Hamidin et al. 2008; Jeong et al. 2012) and thus the use of chlorine as an intervention for reducing microbial contamination on produce should also be evaluated in terms of the toxicological effects of chlorination. This is especially of concern in the agricultural setting because the chlorine concentrations maintained in dump tanks and hydrocooler reservoirs of produce processing facilities are 10-25 times higher than the concentrations used for drinking water treatment (Suslow 2003). Li and Mitch (2018) describe the necessary balance between the minimization of the acute risks of pathogen presence in drinking water and the minimization of the chronic risks of drinking water disinfection byproducts. Similarly, risk assessments should consider the impacts of produce and agricultural water disinfection technologies on both the microbial and chemical risks to produce consumers.

Lastly, any assessment of the risk of salmonellosis incidence resulting from the use of surface water for irrigation has to consider the fact that concentrations of generic *E. coli*, and not specific pathogens, are used to examine microbiological surface water quality. Stine et al. (2005) conducted a microbial risk assessment to determine a concentration of *Salmonella* in irrigation water that would result in an acceptable level of risk of *Salmonella* infection from consuming produce (based on the U.S. Environmental Protection Agency's goal for risk of infection from waterborne pathogens in drinking water). In this risk assessment, depending on the time between the last irrigation event and harvest, different *Salmonella* concentrations in irrigation water were considered acceptable. However, growers and regulators do not measure *Salmonella* concentrations—they measure indicator organisms. Unfortunately, the correlations between indicator organisms and many enteric pathogens are weak and there are likely differences in the relationships between indicator organism concentrations and various foodborne pathogen concentrations (Harwood et al. 2005; Ferguson et al. 2012; Pachepsky

et al. 2016; Payment and Locas 2011; Wilkes et al. 2009; Wu et al. 2011). Thus, further work is necessary to bridge the gap between current understanding of the risks of infection associated with preharvest pathogen concentrations and the body of scientific work that has attempted to estimate the relationship between indicator bacteria and enteric pathogens. This will potentiate the development of indicator bacteria standards that can more effectively reduce the microbial risks of consuming contaminated produce.

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Conflict of Interest

No relevant conflicts of interest were present in this study.

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Table 2-1: Proportion of *Salmonella* positives and Most Probable Number (MPN) of *Salmonella* detected by sample type. For each sample type, the proportion of samples positive for *Salmonella*, the highest concentration detected in one sample of that type as well as the geometric mean of concentrations of all samples of that type are included. Sample types are further stratified by farm. At Farm SC, there were 2 pumping stations for one pond so irrigation system and produce samples are further differentiated by pumping station: SC1 and SC2.

Sample Type		*N*	No. of Positive Samples (% positive)	Highest Concentration Detected (MPN/100 ml)	Geometric Mean of Concentrations (MPN/100 ml)
Water Source	Pond	24	8 (33.3%)	0.37	0.04
	Well Spigot	5	1 (20.0%)	0.055	0.03
Irrigation System	Pivot	6	5 (83.3%)	0.99	0.09
	Pond Spigot	15	4 (26.7%)	0.16	0.04
	Solid Set Sprinkler	3	0 (0.0%)	ND	0.03
	Drip (Pond)	30	7 (23.3%)	0.16	0.03
	Drip (Well)	11	1 (0.09%)	0.055	0.03
Produce		65	2 (0.03%)	1.28*	0.03*
TOTAL		159	28 (17.6%)		

ND = Non-detect
*MPN/produce sample

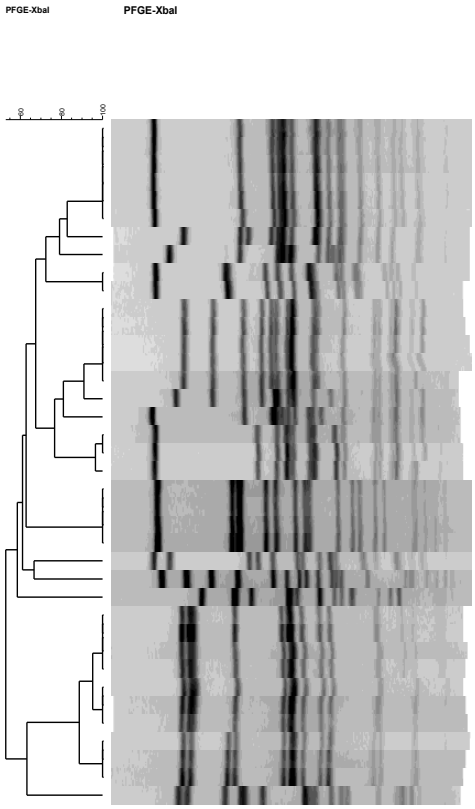
Table 2-2: Detected *Salmonella* serovars. Serovars detected from 38 distinct study isolates selected for serotyping analysis and the incidence of salmonellosis from the specific serovar in Georgia and in the United States.

Serovar	# of study isolates (%)	Incidence in GA in 2014 per 100,000 population (% of cases in GA)*	Incidence in US in 2014 per 100,000 population (% of cases in US)**
Saintpaul	10 (26.3)	0.69 (3.1)	0.31 (2.2)
Muenchen	9 (23.7)	0.60 (2.7)	0.27 (2.0)
Newport	6 (15.8)	3.57 (16.0)	1.39 (10.0)
Mbandaka	4 (10.5)	0.06 (0.3)	0.06 (0.5)
Hartford	2 (5.3)	0.05 (0.2)	0.05 (0.3)
Rough "O"	2 (5.3)	0 (0)	ND
Javiana	1 (2.6)	3.94 (17.7)	0.85 (6.1)
III_16:z10:e,n,x,z15	1 (2.6)	0 (0)	<0.01 (<0.1)
III_60:r:e,n,x,z15	1 (2.6)	0 (0)	<0.01 (<0.1)
Bardo	1 (2.6)	0 (0)	ND
Rubislaw	1 (2.6)	0.15 (0.7)	0.07 (0.5)

* Georgia Department of Public Health Data received through Public Health Information Portal

** National Enteric Disease Surveillance: *Salmonella* Annual Report, 2015 (CDC 2017a)

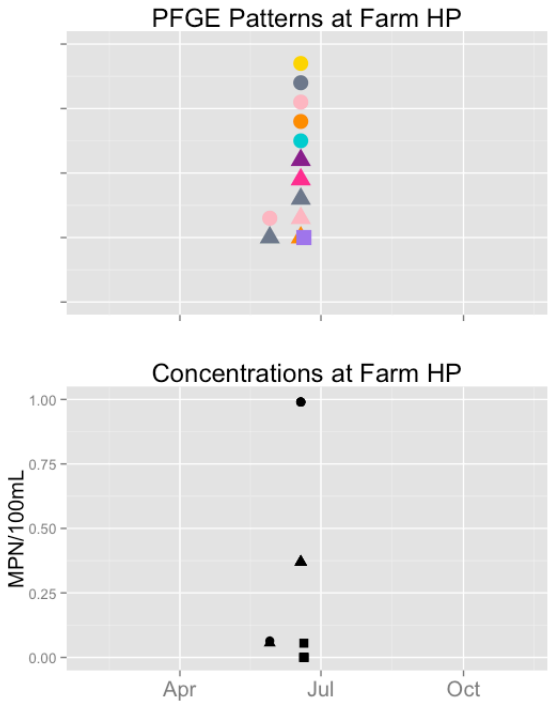
Figure 2-1: Dendrogram of the unique PFGE patterns and serovars of *Salmonella* isolated in this study. Isolate IDs correspond to the sample (number) and isolate from each sample (letter), in cases with >1 isolate/sample. For each isolate, "Pump" refers to the farm where samples were collected; "Rotation" indicates the crop planted in the fields at the time the sample was collected; "Sample" indicates the sample type; "Serovar" indicates the results of the serotyping analysis, and "PFGE Pattern" corresponds to the clustering within this dendrogram. The 38 unique PFGE patterns are shown, of 143 total *Salmonella* isolated in the study, because many PFGE clones were present within the full isolate collection.



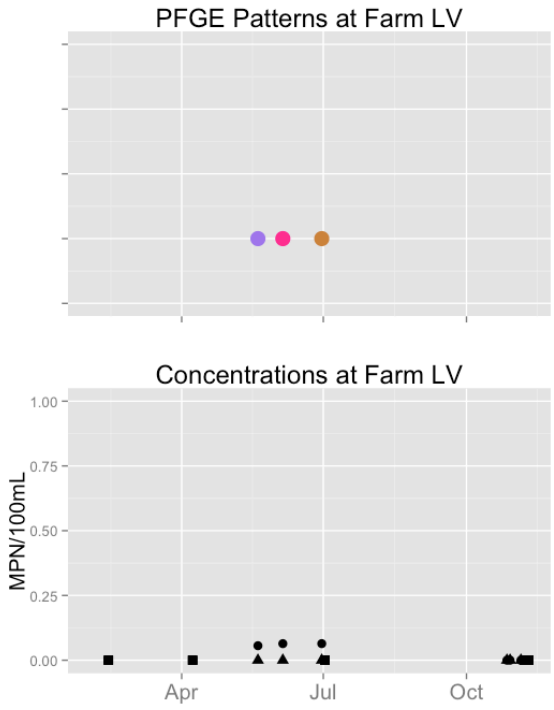
ID	Date	Pump	Rotation	Sample	Serovar	PFGE Pattern
1	05/05/2014	WL	Watermelon	Irrigation	Newport	1
2	05/05/2014	WL	Watermelon	Well	Newport	1
5	05/13/2014	SC1	Squash	Irrigation	Newport	1
6	05/20/2014	LV	Cantaloupe	Irrigation	Newport	1
27	06/20/2014	HP	Cantaloupe	Produce	Rough "O"	1
28-B	07/08/2014	SC1	Cucumber	Produce	Rough "O"	1
28-A	07/08/2014	SC1	Cucumber	Produce	Bardo	2
4	05/13/2014	SC1	Squash	Pond	Newport	3
18	09/23/2014	SC2	Bell peppers	Pond	Hartford	4
19	09/23/2014	SC2	Bell peppers	Irrigation	Hartford	4
14	08/18/2014	SC2	Bell peppers	Pond	Muenchen	5
20	10/08/2014	SC1	Zucchini	Pond	Muenchen	5
21	10/08/2014	SC1	Zucchini	Irrigation	Muenchen	5
23-B	10/08/2014	SC1	Zucchini	Irrigation	Muenchen	5
25	10/20/2014	SC1	Zucchini	Irrigation	Muenchen	5
15	08/18/2014	SC2	Bell peppers	Irrigation	Muenchen	6
26	10/23/2014	SC1	Zucchini	Irrigation	Muenchen	7
3-A	06/18/2014	HP	Cantaloupe	Pond	Muenchen	8
11-B	06/18/2014	HP	Cantaloupe	Irrigation	Muenchen	8
11-E	06/18/2014	HP	Cantaloupe	Irrigation	Muenchen	9
17	08/25/2014	SC2	Bell peppers	Irrigation	Mbandaka	10
22	10/08/2014	SC1	Zucchini	Irrigation	Mbandaka	10
23-A	10/08/2014	SC1	Zucchini	Irrigation	Mbandaka	10
24	10/20/2014	SC1	Zucchini	Pond	Mbandaka	10
3-B	06/18/2014	HP	Cantaloupe	Pond	Rubislaw	11
16	08/25/2014	SC2	Bell peppers	Pond	III_60:r,e,n,x,z15	12
11-A	06/18/2014	HP	Cantaloupe	Irrigation	Javiana	13
9	05/29/2014	HP	Cantaloupe	Irrigation	Saintpaul	14
3-C	06/18/2014	HP	Cantaloupe	Pond	Saintpaul	14
11-C	06/18/2014	HP	Cantaloupe	Irrigation	Saintpaul	14
12	06/18/2014	SC2	Watermelon	Irrigation	Saintpaul	14
7	05/27/2014	SC2	Watermelon	Irrigation	Saintpaul	15
10	06/05/2014	LV	Cantaloupe	Irrigation	Saintpaul	15
3-E	06/18/2014	HP	Cantaloupe	Pond	Saintpaul	15
8	05/29/2014	HP	Cantaloupe	Pond	Saintpaul	16
3-D	06/18/2014	HP	Cantaloupe	Pond	Saintpaul	16
11-D	06/18/2014	HP	Cantaloupe	Irrigation	Saintpaul	16
13	06/30/2014	LV	Cantaloupe	Irrigation	III_16:z10:e,n,x,z15	17

Figure 2-2: Temporal and spatial distribution of PFGE patterns of 38 *Salmonella* isolates from southern Georgia farms: a) HP, b) LV, c) SC, and d) WL. The top graphs display the isolates at each farm by PFGE pattern and sample type (source water, irrigation water, and produce) over the study period (x-axis). For each isolate dot of the top graph, the color and symbol correspond to the PFGE pattern and sample type, respectively. PFGE data from samples associated with both pumping stations at Farm SC were aggregated. The *Salmonella* concentration in samples from which the patterns were isolated is indicated directly below each PFGE figure. For each graph, the x-axis is the time of the study period (May to November 2014).

a)



b)



c)

d)

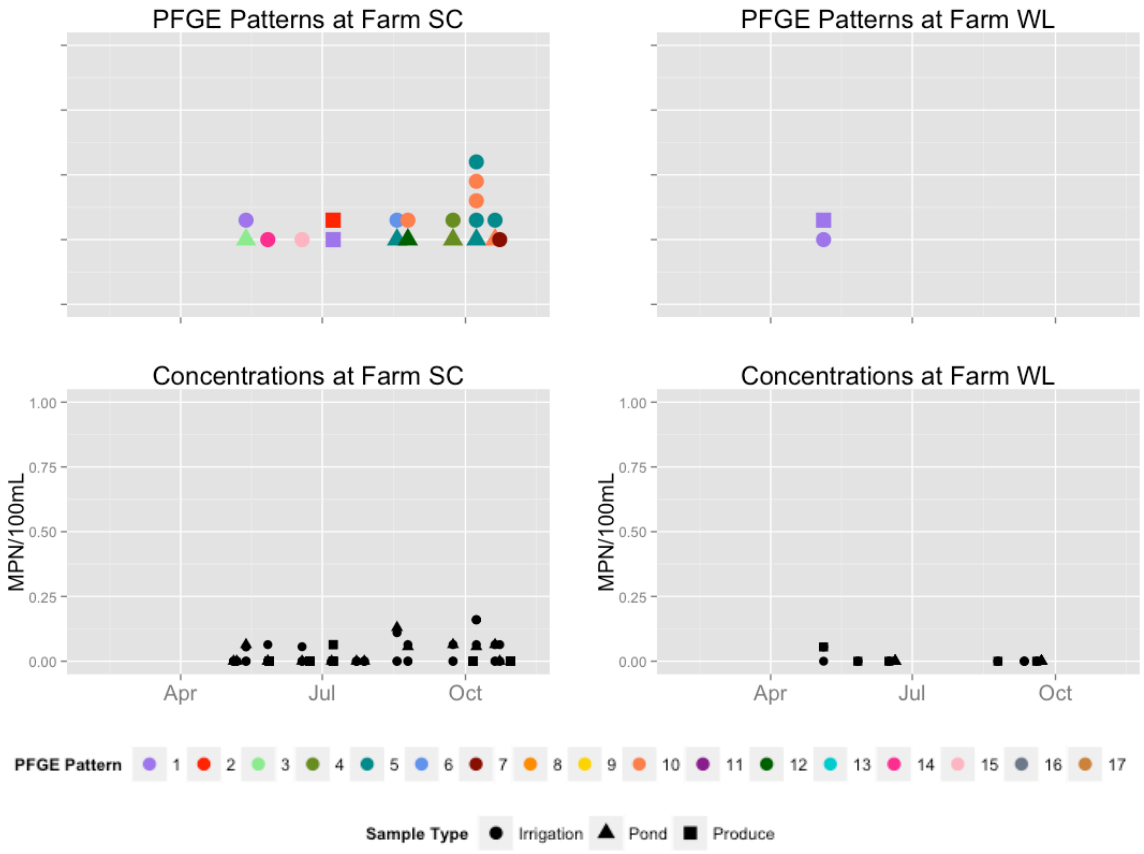

PFGE Patterns at Farm SC
PFGE Patterns at Farm WL
Concentrations at Farm SC
Concentrations at Farm WL
MPN/100mL
1.00
0.75
0.50
0.25
0.00
Apr
Jul
Oct
PFGE Pattern
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Sample Type
Irrigation
Pond
Produce

Table 2-3: Summary of location and sample type for each of the 17 *Salmonella* PFGE patterns and associated serovars detected on farms in southern Georgia. All but two of the patterns matched patterns present in the U.S. Center for Disease Control and Prevention (CDC)'s PulseNet database. Instances when the serovar determination differed between serotyping and PulseNet (PFGE Pattern) or SISTR are indicated in parentheses. If a mismatch is not indicated, then the serovar predicted by PulseNet or SISTR was in accordance with our serotyping analysis. The total number of PulseNet entries for each pattern indicates the prevalence of the pattern in the United States and Georgia.

PFGE pattern	Where identified	Serovar	Total No. of PulseNet Entries for This Pattern in the U.S. (whole database)	Total No. of PulseNet Entries for This Pattern in Georgia* (whole database)	WGS Cluster(s)**
1	Source (WL) Irrigation (WL, SC, LV) Produce (HP, SC)	Newport Newport Rough "O" (Newport[a])	558	1	5
2	Produce (SC)	Bardo (Newport[a])	1	0	5
3	Source (SC)	Newport	4	0	5
4	Source (SC) Irrigation (SC)	Hartford	13	1	9
5	Source (SC) Irrigation (SC)	Muenchen	5	1	2 (1 isolate not in any cluster)
6	Irrigation (SC)	Muenchen	3	0	Not in any cluster
7	Irrigation (SC)	Muenchen	N/A	N/A	8
8	Source (HP)	Newport	46	3	8

	Irrigation (HP)				
9	Irrigation (HP)	Muenchen	N/A	N/A	8
10	Source (SC) Irrigation (SC)	Mbandaka	1	1	10
11	Source (HP)	Rubislaw	1	1	3
12	Source (SC)	IIIb_60:r,e,n,x,z15	2	0	4
13	Irrigation (HP)	Javiana	13	8	6
14	Source (HP) Irrigation (HP, SC)	Saintpaul	471	66	11
15	Source (HP) Irrigation (LV, SC)	Saintpaul	167	17	7
16	Source (HP) Irrigation (HP)	Saintpaul	90	10	1
17	Irrigation (LV)	III_16:z10:e,n,x,z15	1	0	2

[a] serovar through serotyping
*patient sought care in Georgia
**NCBI Pathogen Detection tree accessed February 2, 2018

Figure 2-3: Dendrogram of clades with closely related sequences. Sample type, serovar as determined by serotyping, and PFGE pattern are indicated next to each isolate. Single Nucleotide Polymorphism (SNP) differences between genomes of *Salmonella* isolates are noted between branches of isolates.

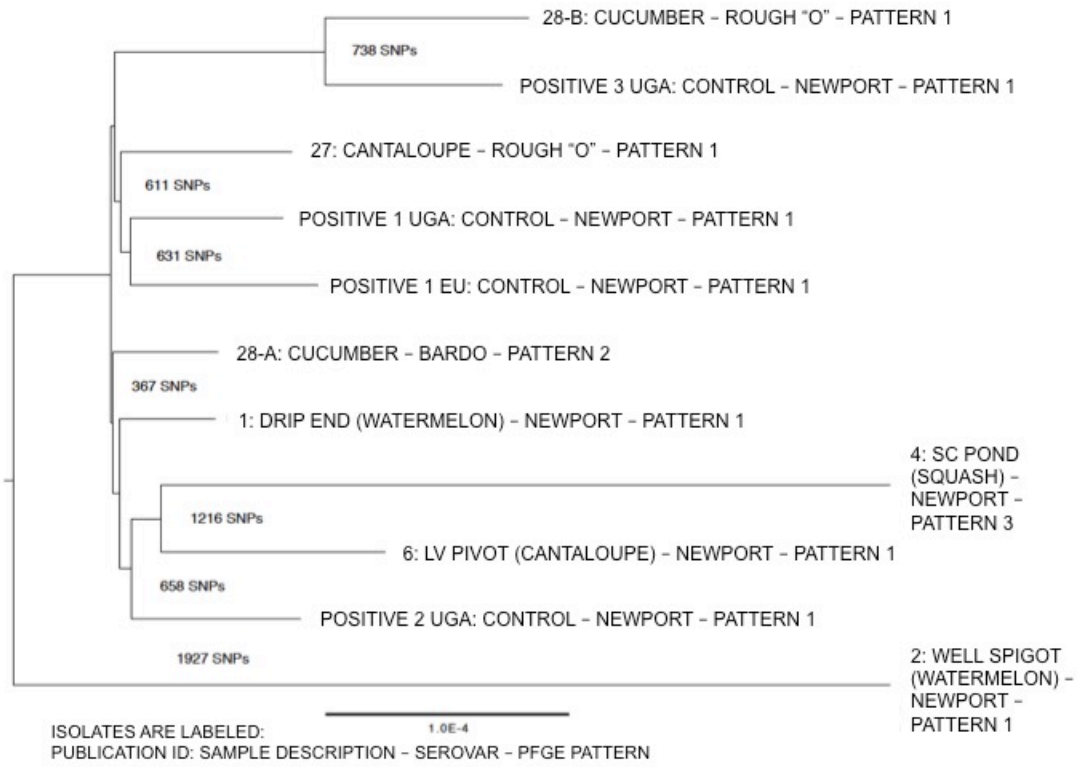
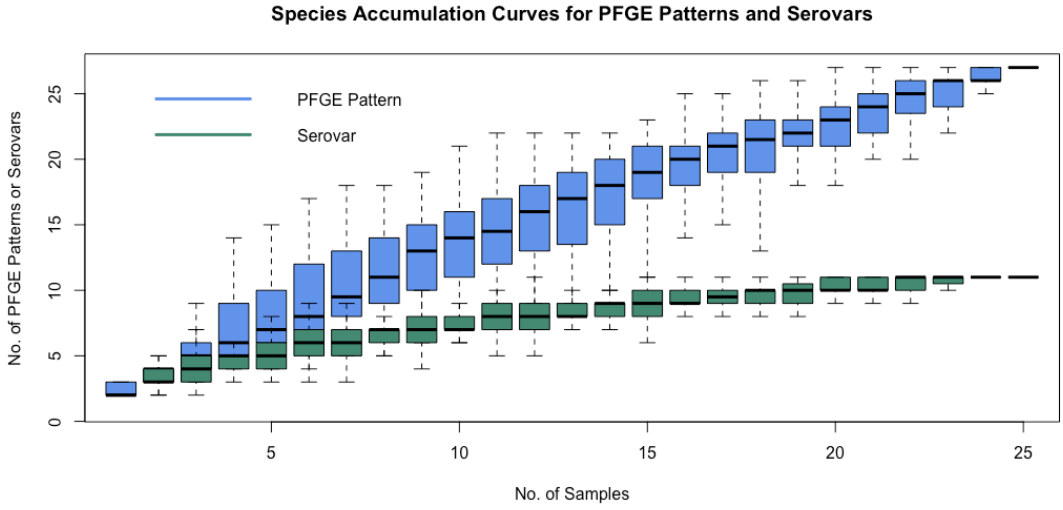


Table 2-4: Comparison of our study samples to *Salmonella enterica* samples in the NCBI Pathogen Detection database using SNP clusters. The database was accessed on February 2, 2018. Study isolates were found in 11 SNP clusters in this database. For each cluster, the number and sources of study isolates are listed. Additionally, the sources, geographic regions, and years of pathogen detection for all cluster isolates are indicated.

		Study Samples		Cluster Samples		
Cluster	Total # in cluster	# of study samples in cluster	Source	Source	Geographic Region	Years
1	11	4	Pond Irrigation	Clinical (4) Environmental (7)	USA USA (SC, GA)	2014-2016
2	19	5	Pond Irrigation	Environmental (19)	USA (GA)	2012-2014
3	12	1	Pond	Environmental (12)	USA (GA)	2011-2014
4	3	1	Pond	Environmental (3)	USA (GA)	2012-2014
5	883	11	Pond Produce Well Irrigation	Clinical (616) Environmental (267): -Produce (41) -Wildlife-related (6)	USA (AL, FL, NY, DC); Canada; UK USA (AK, DE, FL, GA, MD, MI, NC, NY, PA, SC, VA, WA); Peru	1979-2017
6	3	1	Irrigation	Clinical (2) Environmental (1)	USA USA (GA)	2014-2015

7	11	4	Pond Irrigation	Clinical (7) Environmental (4)	USA (FL) USA (GA)	2014-2016
8	13	4	Pond Irrigation	Environmental (13): -Salamander (1)	USA (GA)	2011-2014
9	4	2	Pond Irrigation	Environmental (4): -Trachemys scripta (1)	USA (GA)	2010-2014
10	945	4	Pond Irrigation	Clinical (351) Environmental (594): -Poultry-related (156) -Pork-related (34) -Beef-related (37) -Wildlife-related (12) -Plant-based food (82)	Canada; Taiwan; UK (117); USA (CA, NY, WY) Argentina; Belize; Brazil; Canada; China; Denmark; Egypt; India; Japan; Lebanon; Mexico; Pakistan; Singapore; Syria; Taiwan; Turkey; UK; USA (45 states)	1989-2017
11	165	6	Pond Irrigation	Clinical (22) Environmental (143): -Produce (2) -Wildlife (2)	USA USA (FL, GA, NC)	2004-2017

Figure 2-4: Species accumulation curves of the *Salmonella* PFGE patterns and serovars isolated from farms in southern Georgia. The serovar species accumulation curve has plateaued, indicating that increased sampling is unlikely to uncover additional serovars. In contrast, the PFGE pattern curve continues to increase, indicating the potential for the discovery of more PFGE patterns with additional sampling.

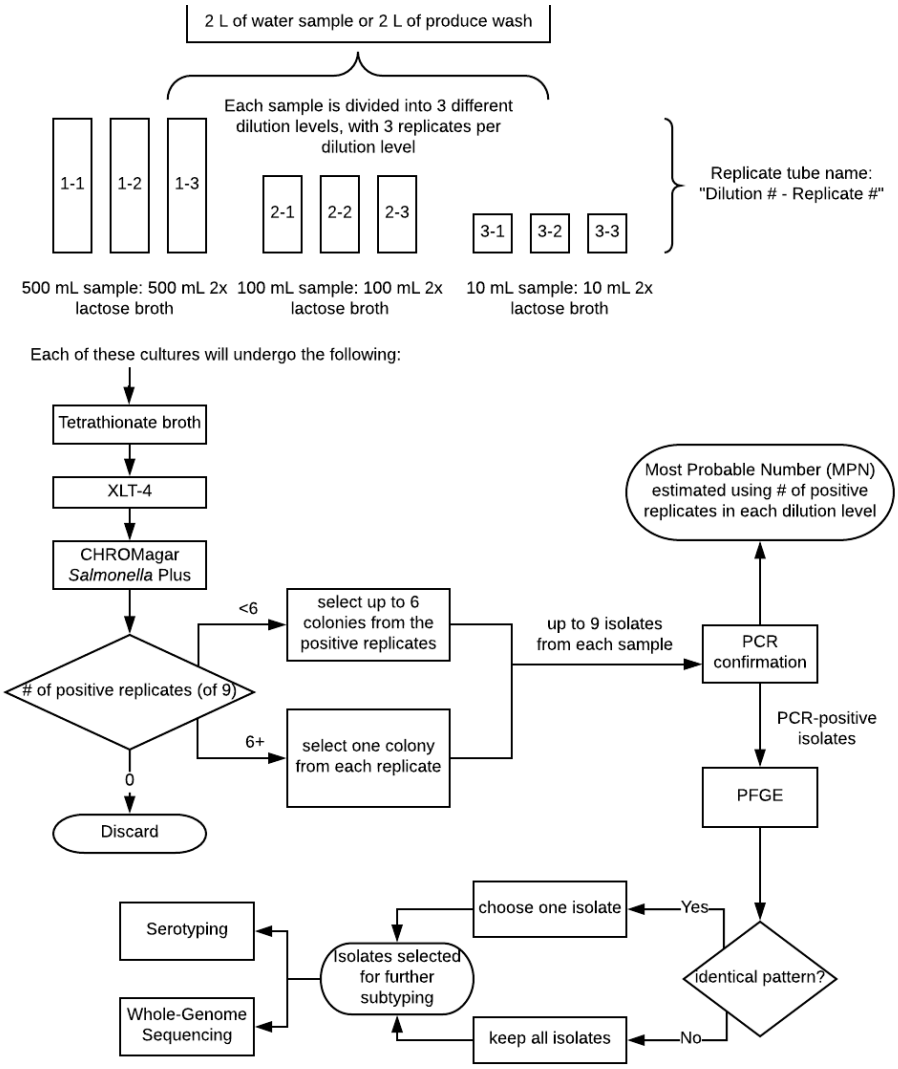


Supplementary Table S2-1: Summary of produce and water samples collected. Thirteen commercial harvests were investigated in this study. With the exception of two harvests, irrigation water sources and irrigation distribution systems were sampled prior to the harvest. For each crop harvest, the harvest date, associated irrigation system and water source, and crop type are listed. For each sample type of a harvest, the units per sample and number of samples collected are also listed.

Harvest Date	**Irrigation System**	**Water Source**	**Crop**	**Sample**	**Units per Sample**	**No. of Samples**
Feb 2014	Pivot sprinkler	Pond	Broccoli	Pond	NA	NA
				Irrigation	NA	NA
				Produce	5 crowns	5
April 2014	Solid set sprinkler	Pond	Mustard Greens	Pond	NA	NA
				Irrigation	NA	NA
				Produce	6 leaves	5
May 2014	Drip irrigation	Pond	Squash	Pond	NA	NA
				Irrigation	NA	NA
				Produce	6 pieces	5
June 2014	Pivot sprinkler	Pond	Cantaloupe*	Pond	2 liters	3
				Irrigation	2 liters	3
				Produce	2 pieces	5
June 2014	Drip irrigation	Well	Cucumber	Well	2 liters	3
				Irrigation	2 liters	6
				Produce	6 pieces	5
June 2014	Drip irrigation	Pond	Watermelon	Pond	2 liters	3
				Irrigation	2 liters	9
				Produce	1 piece	5
July 2014	Pivot sprinkler	Pond	Cantaloupe	Pond	2 liters	3
				Irrigation	2 liters	3
				Produce	2 pieces	5
July 2014	Drip irrigation	Pond	Cucumber*	Pond	2 liters	6
				Irrigation	2 liters	6
				Produce	8 pieces	5
Aug 2014	Drip irrigation	Well	Cucumber	Well	2 liters	3
				Irrigation	2 liters	6
				Produce	8 pieces	5
Oct 2014	Drip irrigation	Pond	Bell Pepper	Pond	2 liters	3
				Irrigation	2 liters	9
				Produce	6 pieces	5

Oct 2014	Drip irrigation	Pond	Zucchini	Pond	2 liters	3
				Irrigation	2 liters	9
				Produce	8 pieces	5
Nov 2014	Solid set sprinkler	Pond	Mustard Greens	Pond	2 liters	3
				Irrigation	2 liters	3
				Produce	30 leaves	5
Nov 2014	Solid set sprinkler	Pond	Mustard Greens	Pond	2 liters	3
				Irrigation	2 liters	3
				Produce	30 leaves	5

Supplementary Figure S2-1: Sample Workflow. This flowchart depicts all of the methods used in this study from sample collection, *Salmonella* isolation, enumeration of *Salmonella* concentrations, PCR confirmation, pulsed-field gel electrophoresis (PFGE), serotyping, and whole genome sequencing. See text for experimental details.



Supplementary Table S2-2: Pairwise comparison of single nucleotide polymorphism (SNP) differences in genomes of *Salmonella* isolates. The number of SNP differences between two isolate genomes is listed. The reference genome for SNP analysis was *S. enterica* AE014613.

	# SNP differences using PARSNP			**# SNP differences using NUCmer**		
CFSAN051024	632			738		
CFSAN051028						
CFSAN051023	501			611		
CFSAN051026		523			631	
CFSAN051025						
CFSAN051030	309			367		
CFSAN051019						
CFSAN051029	688			1216		
CFSAN051022		572			658	
CFSAN051027	1658			1927		
CFSAN051020						

Supplementary Table S2-3: NCBI BioSample accession numbers for *Salmonella* sequences of study isolates. For each study isolate, the sample type, geographic origin of the isolate, and BioSample ID are listed. Publication ID refers to the sample ID as used in Figure 2-1.

Publication ID	**Pump**	**CFSAN ID**	**BioSample ID (Accession Number)**
1	WL	CFSAN051019	SAMN05195914
2	WL	CFSAN051020	SAMN05195915
4	SC1	CFSAN051029	SAMN05195924
5	SC1	CFSAN051021	SAMN05195916
6	LV	CFSAN051022	SAMN05195917
7	SC2	CFSAN051008	SAMN05195903
8	HP	CFSAN051010	SAMN05195905
9	HP	CFSAN051006	SAMN05195901
10	LV	CFSAN050998	SAMN05195893
12	SC2	CFSAN051004	SAMN05195899
13	LV	CFSAN051043	SAMN05195938
14	SC2	CFSAN051035	SAMN05195930
15	SC2	CFSAN051040	SAMN05195935
16	SC2	CFSAN051017	SAMN05195912
17	SC2	CFSAN051013	SAMN05195908
18	SC2	CFSAN051031	SAMN05195926
19	SC2	CFSAN051032	SAMN05195927
20	SC1	CFSAN051036	SAMN05195931
21	SC1	CFSAN051037	SAMN05195932

22	SC1	CFSAN051014	SAMN05195909
24	SC1	CFSAN051016	SAMN05195911
25	SC1	CFSAN051039	SAMN05195934
26	SC1	CFSAN051041	SAMN05195936
27	HP	CFSAN051023	SAMN05195918
11-A	HP	CFSAN051012	SAMN05195907
11-B	HP	CFSAN051033	SAMN05195928
11-C	HP	CFSAN051003	SAMN05195898
11-D	HP	CFSAN051000	SAMN05195895
11-E	HP	CFSAN051034	SAMN05195929
23-A	SC1	CFSAN051015	SAMN05195910
23-B	SC1	CFSAN051038	SAMN05195933
28-A	SC1	CFSAN051030	SAMN05195925
28-B	SC1	CFSAN051024	SAMN05195919
3-A	HP	CFSAN051042	SAMN05195937
3-B	HP	CFSAN051018	SAMN05195913
3-C	HP	CFSAN051005	SAMN05195900
3-D	HP	CFSAN050999	SAMN05195894
3-E	HP	CFSAN050997	SAMN05195892
3-A-dup	HP	CFSAN051001	SAMN05195896
9-dup	HP	CFSAN051002	SAMN05195897
3-C-dup	HP	CFSAN051007	SAMN05195902
7-dup	SC2	CFSAN051009	SAMN05195904

8-dup	HP	CFSAN051011	SAMN05195906
POS 1 EU		CFSAN051025	SAMN05195920
POS 1 UGA		CFSAN051026	SAMN05195921
POS 2 UGA		CFSAN051027	SAMN05195922
POS 3 UGA		CFSAN051028	SAMN05195923

Supplementary Figure S2-2: SNP cluster (in red) in NCBI Pathogen Detection database. This cluster contains three produce isolates from this study.

CHAPTER THREE

Salmonella Survival in Soil and Transfer onto Produce via Splash Events

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Abstract

During rainfall and irrigation events, pathogens in soil can be transported from fields to crops through surface water runoff. One produce contamination route that is not often explored is the transfer of pathogens to edible portions of crops via splash water. We report here on the results from multiple field and microcosm experiments examining the potential for *Salmonella* contamination of produce crops via splash water, and the impact of soil moisture content on *Salmonella* survival in soil and concentration in splash water. In our field and microcosm experiments, we were able to detect *Salmonella* for up to 8-10 days in soil and on produce. *Salmonella* and suspended solids were detected in splash water at heights of up to 80 cm. Soil moisture conditions antecedent to the splash event had an impact on detection of *Salmonella* on crops after splash events—*Salmonella* concentrations detected on produce following rainfall were significantly higher on wet plots than on dry plots (geometric mean difference = 0.43 CFU/g; $p = 0.04$). Similarly, concentrations of *Salmonella* in splash water in wet plots trended higher than concentrations from dry plots (geometric mean difference = 0.67 CFU/100 ml); $p = 0.07$). These results indicate that splash transfer of *Salmonella* from soil onto crops can occur and that antecedent soil moisture content may mediate the efficiency of microbial transfer. Splash transfer of *Salmonella* may therefore pose a hazard to produce safety and thus, the potential for the risk of splash should be further explored in agricultural regions where *Salmonella* and other pathogens are present in soil.

Introduction

The Centers for Disease Control and Prevention estimate that there are 48 million foodborne illnesses every year in the United States (Scallan et al. 2011a; Scallan et al. 2011b). Estimates suggest that produce consumption is responsible for nearly half of all foodborne illnesses in the United States (Painter et al. 2013). Produce can become contaminated at numerous points in the food production chain: during the production, processing, distribution, and preparation steps (Bartz et al. 2017). Preharvest contamination can occur through contact with pathogens in irrigation water, soil, soil amendments, and wildlife feces (Barak and Liang 2008; Beuchat 2006; Erickson 2016; Gelting et al. 2011; Park et al. 2012; Strawn et al. 2013b), and can persist through postharvest steps, resulting in cross-contamination of downstream processes in the food production chain (Jung et al. 2014; Murray et al. 2017; Olaimat and Holley 2012; Tomás-Callejas et al. 2011; Warriner et al. 2009).

Certain pathogens, such as *Escherichia coli*, *Salmonella* spp. and *Listeria* spp., can survive and persist in soil, water, and on edible portions of crops and in turn, pose a hazard to produce safety (Danyluk et al. 2008; Erickson et al. 2014; Holley et al. 2006; Islam et al. 2004; Islam et al. 2005). Pathogens in surface or groundwater can be transferred to crops during irrigation and pathogens in untreated biological soil amendments can contaminate crops through direct contact or transport into irrigation water sources (Franz and van Bruggen 2008; Jacobsen and Bech 2012). There is also evidence that rainfall is associated with crop contamination (Strawn et al. 2013a; Weller et al. 2015a).

One potential mechanism of rainfall contamination of crops is splash transfer of human pathogens from soil onto crops during rainfall and irrigation events. This mechanism is more often explored in the context of crop contamination with plant pathogens (Jenkinson and Parry 1994; Madden 1997; Penet et al. 2014) than as a preharvest produce safety concern. Recently, two field studies have provided evidence of splash transfer of pathogenic and non-pathogenic *E. coli* from simulated feces onto crops (Atwill et al. 2015; Weller et al. 2017), pointing to point source contamination from wildlife intrusion or untreated manure application as a potential produce safety risk. However, pathogen transport during rainfall or irrigation is driven by numerous factors, such as soil type, soil moisture, microbial adhesion to soil particles, and water droplet impact (Bradford et al. 2013).

The impact of soil moisture content antecedent to rainfall has previously been explored in the context of soil infiltrability and sediment transport via splash and runoff (Liu et al. 2011; Truman et al. 2011). Generally, higher antecedent soil moisture is associated with the greater impermeability of soil and the subsequent increase in overland flow. Higher antecedent soil moisture is also associated with increased microbial transport (Callahan et al. 2017; Tallon et al. 2007; Van Elsas et al. 1991). Even though the impacts of antecedent soil moisture on soil splash, runoff, and microbial transport have been investigated, there is little known about the impact of soil moisture on the splash transfer of human pathogens from soil onto fresh produce.

In this study, we investigated the potential for splash transfer of *Salmonella* from soil onto crops during rainfall and irrigation events. Additionally, because estimating the risks of fresh produce contamination may require a soil- and location-specific understanding of splash

transfer in the production environment, we investigated the impact of antecedent soil moisture conditions on the efficiency of splash transfer in the agriculturally productive region of southern Georgia, where *Salmonella* is regularly detected in the environment (Antaki et al. 2016; Haley et al. 2009; Harris et al. 2018; Li et al. 2014; Luo et al. 2015; Maurer et al. 2015). A prior study observed splash transfer of *Salmonella* in small-scale experiments in a greenhouse (Cevallos-Cevallos et al. 2012) and our experiments served to validate and expand upon these results with large-scale experiments in fields. Additionally, we studied the survival of *Salmonella* in soil and on crops during the fall growing season. This study aims to inform growers of potential risks from splash associated with overhead sprinkler irrigation and heavy rainfall events, in order to provide useful information to guide management decisions toward reducing the risk of fresh produce contamination via splash water.

Materials and Methods

We carried out several field and experimental studies (microcosm experiments, field experiments, soil surveillance), in order to inform the goals of this study from multiple angles. The overview of experiments can be found in Figure 3-1. Data from each of these studies complement one another.

Microcosm Experiments

We carried out microcosm experiments to understand the dynamics of *Salmonella* survival in soil under laboratory conditions. Three wet microcosms and three dry microcosms were created using soil from University of Georgia's Horticulture Farm in Tifton, Georgia, which also served as the site of the final two field experiments (see below). For each microcosm, 1.5 kg of soil was lightly packed for a bulk density of 1.3 g/ml. Microcosms were

constructed using open 3.1 liter Glad® plastic containers (Glad Products Company, Oakland, CA, USA). Container bottoms were perforated to allow for water drainage. The saturation point and field capacity of these microcosms were 20% and 11%, respectively, using a gravimetric method of estimating soil moisture content. Wet microcosms were brought to near-saturation by adding 200 ml of sterile deionized water. No water was added to the dry microcosms. A 100 ml inoculum (3 log CFU/ml) of avirulent *Salmonella* Typhimurium (χ3985 Δcrp-11, Δcya-12), labeled with a green fluorescent protein (GFP) plasmid containing an ampicillin-resistant marker (Erickson et al. 2018a, b), was evenly applied to the surface of three wet and three dry microcosms. One wet microcosm and one dry microcosm were also constructed as previously described but not inoculated—they were used for soil moisture analyses. Three soil samples were collected from the microcosms on Days 0, 2, 4, 6, and 8, using sterile lab spoons. Sampling sites were selected to collect a sample set representative of the microcosm surface. Soil samples were composed of approximately 15 g of soil and were washed with 30 ml of 0.1% weight per volume (w/v) buffered peptone water (BPW; BD BBL, Franklin Lakes, NJ, USA). Soil samples were shaken for 30 seconds, massaged for 60 seconds, and shaken again for 30 seconds. Aliquots of 100 µl were plated onto xylose-lysine-tergitol-4 (XLT-4) agar (BD Difco™) with ampicillin (100 µg/ml; Thermo Fisher Scientific, Waltham, MA, USA) and plates were incubated at 37°C for 24 hours. Fluorescent colonies were visualized using UV light. On each sampling day, one soil sample was collected from the dry and wet microcosms set aside for moisture analyses and analyzed using a gravimetric method for water content. Following sampling, wet microcosms were watered up to their saturation point so that watering would not result in *Salmonella* leaching out of the container.

Field Experiments

We carried out a series of field experiments to understand splash transfer of *Salmonella* under more realistic conditions than those provided by the microcosm experiments. The first experiment was carried out on three fields of a vegetable farm near Tifton, Georgia. The final two experiments were carried out at University of Georgia's Horticulture Farm in Tifton, Georgia.

Field Experiment 1 (Overhead irrigation and natural rainfall): The study site for this experiment comprised a vegetable farm with three fields, planted with broccoli, mustard greens, and cantaloupe (Figure 3-2). Fields were irrigated from a 0.57-hectare pond. Two of the fields (broccoli, cantaloupe) were irrigated by a center pivot irrigation system while the third was irrigated by a solid-set irrigation system (mustard greens). Six overhead irrigation events (December 2013 to June 2014) and three natural rainfall events (January 2014 to April 2014) were studied. For each event, due to sampling feasibility, five sampling locations (nodes) were selected to represent the area of each field. At each node, five aluminum pans were installed at heights of 5, 10, 20, 40, and 80 cm. Under the pivot, the pans were deployed to capture samples from 5 different spans of the pivot. Under the solid set system, we selected two blocks of the field with the most uniform coverage by the sprinklers, as indicated from aerial photographs. Within these two blocks, the locations of the five nodes were randomly chosen using a random number generator for the number of steps to take within each block. The location of each sampling pan was marked with a flag and sampling pans were reinstalled in the same location at subsequent sampling events. However, the height of the sampling pan at that location was randomly selected for each event using a random number generator. The sampling pans were installed in the study fields immediately

before an irrigation event and used to capture irrigation water and water splashing from the soil surface during the irrigation event. A photo of the installation under the solid set sprinkler is shown in Figure 3-3.

After the irrigation event was completed (or the pivot had moved past the sampling pans), water and any solids collected in the sampling pans were poured into sterile sample bottles. At the laboratory, the replicates from each height were composited, and one composite sample was analyzed per height.

Produce samples were collected from each field at the end of each growing cycle at randomly selected distances and directions from the sampling pan nodes. A duplicate sample was collected at one of the nodes for a total of six samples from each field. The edible portions of crops were collected using sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA). The number of produce units in each sample was as follows: two units for cantaloupe, six crowns for broccoli, and six leaves for mustard greens.

Splash water samples were analyzed for *Salmonella*, *E. coli*, total coliforms, and total suspended solids and produce samples were analyzed for *Salmonella*. *E. coli* and total coliform were quantified using IDEXX Quanti-Tray/2000 System with Colilert Reagent (IDEXX Laboratories, Inc., Westbrook, ME, USA). *Salmonella* concentrations were quantified using a culture-based most probable number method as described by Luo et al (Luo et al. 2014). Produce samples were first washed with 2 liters of 1% w/v BPW. Then, both produce washes and splash water samples were enriched with lactose broth and incubated at 37°C for 24 hours. Then, sample aliquots were added to *Salmonella*-selective tetrathionate broth with

iodine. Cultures were streaked onto XLT-4 agar and CHROMagar™ *Salmonella* Plus agar (CHROMagar™ Microbiology, Paris, France). To extract DNA from samples presumed positive by XLT-4 and/or CHROMagar™ *Salmonella* Plus, each *Salmonella* colony was placed in 200 µl of molecular grade water and boiled at 100°C for 10 minutes to lyse the cells. After 10 minutes, samples were cooled on ice and then centrifuged at 15,000 g for 5 minutes. The supernatant was removed and stored at -20°C. DNA extractions were used as template for 25 µl PCR reactions containing 10 µl of 2x Promega Master Mix (Promega, Madison, WI), 5 µl each of the forward and reverse primers at 10 µM concentration, *invA*-1 (5'-ACAGTGCTCGTTTACGACCTGAAT-3') and *invA*-2 (5'-AGACGACTGGTACTGATCGATAAT-3'), and 5 µl of template DNA (Chiu and Ou 1996). Samples were also analyzed for total suspended solids using the gravimetric method in which water samples were filtered and filters were dried at 100°C.

Field Experiment 2 (Rainfall simulation): Because we simulated rainfall with irrigation system water in Field Experiment 1, we were unable to rule out irrigation water contamination as a source of splash water contamination. Because of this, another experiment was conducted from October 31 to November 18 in 2016 using ampicillin-resistant GFP-labeled *Salmonella*, described above for the microcosm experiments. The experiment was conducted on the University of Georgia's Tifton campus Horticulture Farm. Eight cabbage plots (0.5 m x 6 m) were inoculated with 300 ml (7 Log CFU/ml) culture of the GFP-labeled *Salmonella*. The inoculum concentration was chosen to reflect similar concentrations used by researchers of other splash studies (Cevallos-Cevallos et al. 2012; Monaghan et al. 2012). The inoculum was evenly distributed throughout the plot on soil and crops using a 1-gallon plastic tank sprayer (Scotts Miracle-Gro Company, Marysville, OH,

USA). Half of the plots were designated as 'wet plots' and the other half of the plots were designated as 'dry plots.' Wet plots were irrigated every 2 days with a solid set sprinkler while dry plots were covered with plastic sheeting during irrigation. No rainfall occurred throughout the experiment.

Soil and produce samples were collected prior to inoculation to serve as negative controls. At each sampling event, three soil samples and three produce samples were collected from each plot. Sample sizes and volumes were based on prior studies of *Salmonella* survival in soil and on produce (Cevallos-Cevallos et al. 2012; Islam et al. 2005; Tomás-Callejas et al. 2011). Soil and produce samples were collected immediately following inoculation, and every two days for 8 days, for a total of 5 sampling events (days 0, 2, 4, 6, 8) to mirror the sampling schedule in the Microcosm Experiment. Each produce sample consisted of three cabbage leaves, making sure to sample both the inner and outer leaves. Leaves were cut with sterile knives 2.5 cm above the base of the plant. Each soil sample was a composite of two separate samples collected directly adjacent to each other. During each sampling event, three sampling sites were selected to obtain a representative sample set. Soil samples were collected with a steel auger 2.5 cm in diameter at depths of approximately eight cm. The auger was sterilized between each sample with 70% ethanol.

Due to the observed decay of *Salmonella* in soil from both treatment types by day 8 in the Microcosm Experiment, plots were re-inoculated on day 9, prior to the rainfall simulation, with ampicillin-resistant GFP-labeled *Salmonella* (at original inoculation concentration of 7 log CFU/ml). Soil and produce samples were collected following re-inoculation.

On day 9, a plot-scale rainfall simulator was used to simulate a 30-minute rainfall event at an intensity of 110 mm/hour, similar to the intensity tested by Cevallos-Cevallos et al. (Cevallos-Cevallos et al. 2012). The 3 m tall oscillating nozzle simulator covered a plot area of approximately 3 m x 3 m. The use of this rainfall simulator has been documented previously in other rainfall studies in Tifton, Georgia (Frauenfeld and Truman 2004; Truman et al. 2007; Truman et al. 2011). Prior to the simulation, sterile aluminum pans were installed at varying heights (5 cm, 10 cm, 20 cm, 40 cm), 30 cm away from the crops and directly outside the 3 m x 3 m plot area so that pans would collect splash and not water directly from the simulator. Water from the simulation was collected from the pans in sterile 25-liter containers.

Samples were placed on ice and analyzed within 24 hours of collection. Soil samples were washed with 30 ml of 0.1% w/v BPW and plated onto XLT-4 agar with ampicillin (100 μg/ml) as previously described. Produce samples were washed with 100 ml of 0.1% w/v BPW and washed as previously described. The full wash volume (100 ml) was filtered through a 0.45 μm MF Millipore membrane filter (Millipore Sigma, Darmstadt, Germany). Splash water samples (of varying volumes depending on turbidity) were also filtered through the 0.45 μm membrane filter. Filters were plated onto XLT-4 agar with ampicillin (100 μg/ml).

All plates were incubated at 37°C for 24 hours. Fluorescent green colonies were visualized using UV light.

Field Experiment 3 (Natural rainfall): In Field Experiment 2, we assessed *Salmonella* survival in soil and on produce as well as *Salmonella* contamination of splash water but we were unable to determine whether *Salmonella* transfer via splash water could ultimately result in produce contamination because we inoculated the produce to assess survival under different rainfall conditions. In Field Experiment 3 (December 6-17, 2018), which was also conducted on the University of Georgia's Tifton campus Horticulture Farm, we examined the potential for splash transfer in both splash water and on produce. One dry plot (0.6 m x 6.7 m) and one wet plot (0.6 m x 6.7 m) were analyzed in this experiment. Each plot had two rows of produce 30 cm apart: one row of kale and one of collard greens. Dry plots were kept dry using plastic sheeting for three days prior to the rainfall event. Wet plots did not have plastic sheeting and were subject to 7.1 mm of natural rainfall in the day prior to the rainfall event.

Approximately one hour before the natural rainfall event, for each plot, 7 log CFU/ml of ampicillin-resistant GFP-labeled *Salmonella* culture was applied as described above to the soil between the rows, making sure to avoid the leaves of the kale and collard greens. The inoculum was applied one hour before the natural rainfall event in order to allow the inoculum to equilibrate in and distribute through the soil. This waiting period has previously been used in a study of bacterial distribution in soil (Monaghan et al. 2012). While 300 ml of inoculum had been applied in Field Experiment 2, the three-fold increase in length of the plots in this experiment necessitated an inoculum volume of 1 liter. Similar to the previous field experiment, six kale samples and six collard greens samples were collected from each plot post-inoculation and before the rainfall event to examine the presence of contamination. Three soil samples (~100 g each) were collected from each plot using sterile

plastic scoops. Each sample contained three to four leaves, depending on leaf size. Ten aluminum splash pans were set up at each plot: three pans were installed 5 cm above the ground, with one in between the rows, one outside of the bed (approximately 30 cm from the inoculation site), and one further outside of the bed (approximately 60 cm from the inoculation site); three pans were installed 10 cm above the ground with the same configuration as the pans at 5 cm above ground; two pans were installed 20 cm above the ground between the rows; two pans were installed 40 cm above the ground between the rows. Splash pan configurations can be found in Figure 1.

During the experiment, there was approximately 7.5 mm of rain over a two-hour period. This represented a light to moderate rainfall event (median level for a 24-hour period in this region is 6.1 mm). Splash water from pans was transferred to sterile 1-liter bottles. From each plot, six kale samples and six collard greens samples were collected immediately following the initial rainfall event and one hour after the initial rainfall event in order to assess whether the drying of leaves would impact *Salmonella* concentrations. Three soil samples were collected following the initial rainfall event. Five and ten days after the rainfall event, six kale, six collard greens, and three soil samples were collected from each plot. All samples were placed on ice and analyzed within 24 hours of sample collection.

Soil samples were washed with 100 ml of 0.1% w/v BPW. Soil, produce, and splash samples were processed as previously described.

Throughout Field Experiments 2 and 3, soil moisture content was analyzed using a gravimetric method in which soil samples were dried at 100°C. In Field Experiment 1,

samples were assessed for total suspended solids, while in Field Experiments 2 and 3, samples were assessed for turbidity using a Hach 2100Q portable turbidimeter (Hach Company, Loveland, CO, USA).

Statistical Analysis

Non-parametric Wilcoxon rank-sum tests were used to compare *Salmonella* concentrations from crops and soil between dry and wet plots, and to compare soil moisture content between dry and wet soil in the Microcosm Experiment and Field Experiments 2 and 3. Results with *p*-values less than 0.05 were considered significant.

The die-off rates of *Salmonella* in soil were estimated by fitting a first-order decay model, as described by Chick's Law:

$$C_t = C_0 e^{-kt}$$

where C_t = is the *Salmonella* concentration (CFU/g) at time t, C_o is the initial *Salmonella* concentration, k is the die-off rate constant (day^{-1}) and t is the time since the initial inoculation event (days). Coefficients from log-linear regressions of this decay model were used to estimate die-off rate constants for *Salmonella* in soil. All analyses were conducted in R 3.1.3 (R Core Team 2015).

Soil Surveillance

Finally, we carried out soil surveillance across southern Georgia, in order to gain a sense of the prevalence of *Salmonella* in farm soils in the region. All sampling was conducted in October 2017. A total of 120 soil samples were collected using sterilized plastic spoons from

eight farms at University of Georgia research and education centers located across southern Georgia. At each farm, fifteen sampling sites were chosen to represent the large area of the farms. One sample (~25 g) was collected at each sampling site. Farm locations and are indicated in Figure 3-4.

Soil samples were placed on ice and analyzed with 24 hours of collection. Ten grams of soil were washed with 100 ml of 1% w/v BPW and cultured with a method similar to that of the produce samples. DNA was extracted from presumptive positives using the boiling method previously described in Field Experiment 1.

Another ten grams of soil were washed with 100 mL of 1% w/v BPW. From each 100 ml wash, 50 ml aliquots were filtered through 0.47 um filters and filters were used for DNA extractions with the Qiagen DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA). DNA samples from the culture-based method and the commercial extraction kit method were analyzed for *Salmonella* using PCR targeting the *invA* gene as previously described.

Results

Soil Salmonella *Concentrations*

There were no significant differences in *Salmonella* survival between dry and wet soils in any of the microcosm or field experiments, despite differences in soil moisture content between dry and wet soil for the Microcosm Experiment ($p = 0.003$) and Field Experiment 2 ($p = 0.01$) (Supplementary Table S3-1). By day 10, *Salmonella* could not be detected in soil in all experiments and in Field Experiment 3, a decay of 3-4 $\log_{10}$ in *Salmonella* concentrations (CFU/g) was observed over ten days (Figure 3-5 and Supplementary Table S3-2). *Salmonella*

die-off rate constants for dry soils for the microcosm- and field-based experiments ranged from 0.08-0.70 CFU/g/day while rate constants for wet soils ranged from 0.25-0.89 CFU/g/day (Table 3-1).

Splash Transfer

There was evidence of transfer of soil particles (Figure 3-6) and soil bacteria (Table 3-2, Figure 3-7, Supplementary Table S3-3) through splash water in all three field experiments. In Field Experiment 1, *Salmonella*, *E. coli*, and total suspended solids (TSS) were detected at heights of up to 80 cm during overhead sprinkler irrigation events. During rainfall events in Field Experiment 1, *E. coli* and TSS were detected at heights of 5-10 cm and 80 cm, respectively. *Salmonella* was not detected in rainfall splash. The concentration of TSS in splash water was generally attenuated at increasing heights (Figure 3-6). In all cases, splash from irrigation water had higher TSS than from rainwater. In Field Experiment 3, *Salmonella* concentrations also decreased with increases in splash pan height, and *Salmonella* could be detected in splash water at heights of up to 40 cm. Furthermore, *Salmonella* could be detected in splash water at horizontal distances of up to 30 cm (Figure 3-7). *Salmonella* concentrations in splash water on wet plots (at 5 cm: 21.14 CFU/100 ml) were universally greater than concentrations in splash on dry plots (at 5 cm: 1.14 CFU/100 ml) but this difference in concentration was not statistically significant ($p = 0.07$).

In Field Experiment 3, we examined contamination of produce with *Salmonella*, in addition to the presence of soil particles and contaminants in splash water, as in Field Experiments 1 and 2. Produce contamination was observed in Field Experiment 3 in both dry and wet plots, but contamination levels were greater in the wet plots (geometric mean: 0.24 CFU/g)

than in the dry plots (geometric mean: 0.04 CFU/g). This difference in produce contamination levels was statistically significant ($p = 0.04$).

Produce Salmonella *Survival*

In Field Experiment 3, the geometric means of *Salmonella* concentrations on produce on dry and wet plots immediately after the rainfall event were 0.04 CFU/g and 0.24 CFU/g, respectively. Within one hour of this sample collection, the *Salmonella* concentrations on produce in the dry and wet plots were both 0.04 CFU/g. In Field Experiments 2 and 3, *Salmonella* contamination levels decreased over time and were unable to be detected by day 10 (Figure 3-5). There were no significant differences in *Salmonella* concentrations on produce from dry and wet plots in either experiment.

Soil Surveillance

Of 105 soil samples collected across seven farms, *Salmonella* DNA was detected in one sample from a University of Georgia farm near Tifton, Georgia.

Discussion

This study provides evidence of splash transfer of soil particles and *Salmonella* from soil onto crops, especially under wet conditions. We used a series of experiments to examine the possibility of splash transfer from soil to crops, with increasing specificity. First, Field Experiment 1 established that splash occurs, based on presence of total suspended solids in splash pans at heights up to 80 cm. *Salmonella* and *E. coli* were also detected in sampling pans, suggesting that bacteria could be transported through splash water. Second, Field Experiment 2 established that *Salmonella* contamination in fields could be detected in splash

water during heavy rainfall events. Field Experiment 3 demonstrated that *Salmonella* contamination in soil could be detected not only in splash water, but also on produce. Furthermore, *Salmonella* concentrations in splash were higher in wet plots vs. dry plots, indicating that the transfer of *Salmonella* may be augmented by soil moisture content.

While the possibility of *Salmonella* contamination in irrigation water could not be ruled out in Field Experiment 1, in subsequent experiments, controls were implemented to ensure that *Salmonella* in splash water resulted from splash contamination. In these subsequent experiments, *Salmonella* could be detected in splash water at heights of up to 40 cm. *Salmonella* presence in splash water at these heights indicates the potential for the contamination of crops that mature within 40 cm from the ground, such as leafy greens and cantaloupe. The detection of pathogens in splash water in this study is consistent with studies of plant pathogens in soil (Boyer 2008; Madden 1997; Penet et al. 2014). In these studies, pathogens in soil are dislodged through the impact of rainfall and are subsequently dispersed onto crops—this may also be the mechanism by which *Salmonella* was dispersed in this study. In one study of plant pathogens, splash dispersal of a fungal plant pathogen was observed at distances of up to 60 cm (Jenkinsen and Parry 1994). In contrast, in a study of *Salmonella* transport through splash by Cevallos-Cevallos et al., *Salmonella* could only be detected at heights of 5 cm. In this study by Cevallos-Cevallos et al., vertical dispersal was assessed in areas 9 cm in horizontal distance from the inoculation site while in our study, splash pans were installed directly over the inoculation site. Moreover, the previous study used a 1 ml culture in a petri dish as the source of *Salmonella* while we distributed 300 ml to 1 L of inoculum evenly throughout the lengths of the plot (up to 6.7 m in Field Experiment 3).

The volume and distribution of *Salmonella* on our plots likely increased the probability of salmonellae in splash to be captured in our sampling pans.

Our results suggesting that increased soil moisture content may result in greater concentrations of *Salmonella* in splash water provide new information relevant for understanding the risks associated with splash effects. In studies of runoff, there is evidence that contaminants can be transported by detaching from soil particles into runoff or moving with soil particles into runoff (Abu-Ashour and Lee 2000). Fields with saturated soil are more prone to flooding and overland flow, which may have resulted in the resuspension of salmonellae (in free form or adsorbed to soil particles) in the pooled water at the soil surface. Resuspended salmonellae at the soil surface might have been more effectively transported in splash water than salmonellae adsorbed to soil particles below the soil surface. Interestingly, our results of higher *Salmonella* concentrations in splash water in wet plots are inconsistent with results from studies of soil erosion, where increases in soil moisture are associated with decreased sediment loss in runoff and splash (Truman and Bradford 1990; Truman et al. 2011). In studies of soil erosion, high soil moisture content can also result in surface sealing. Surface sealing during rainfall occurs when droplets disaggregate soil clumps and smaller soil particles fill in pore space between larger particles (Fohrer et al. 1999; Mualem et al. 1990). In addition to hindering water infiltration, surface sealing may have prevented salmonellae from infiltrating. Following inoculation, salmonellae may have traveled to greater depths in dry soil than in wet soil and thus, resulted in higher concentrations of *Salmonella* on top of the soil in wet plots. Thus, even though surface sealing and increased soil moisture content are associated with reduced soil splash, surface sealing may have contributed to the localization

of the inoculum at the soil surface and in turn, the higher concentrations at the surface of wet plots may have resulted in higher *Salmonella* concentrations in the splash water.

Notably, *Salmonella* in splash water could be detected even during moderate rainfall, indicating the efficacy of intermediate intensity droplets in either dislodging *Salmonella* from soil particles or serving as a vehicle for contaminated soil particles. This study also assessed myriad events that could result in the generation of splash water. It explored the differential impacts of irrigation, natural rainfall, and simulated rainfall events on splash transfer potential. These events ranged in volume and intensity. The natural rainfall assessed in Field Experiment 3 represented a moderate rainfall event (greater than median 24-hour rainfall amount in this region) while the simulated rainfall in Field Experiment 2 represented an extreme rainfall event (greater than 24-hour rainfall at the 90^{th} percentile in this region).

Salmonella concentrations in soil decreased by days 8-10 regardless of soil moisture content. The relatively minor role of soil moisture content in mediating *Salmonella* decay is consistent with other studies (Danyluk et al. 2008; Underthun et al. 2018). Prior studies have shown a wide range in *Salmonella* survival and persistence in manure-amended soil—one study was able to detect *Salmonella* at day 332 (Islam et al. 2004; Nyberg et al. 2014; Semenov et al. 2009; You et al. 2006). In the Microcosm Experiment, we stopped analyzing the microcosms by day 10 because they served to inform the development of our field experiments and we stopped when concentrations became very low. Thus, the Microcosm Experiment was unable to assess long-term survival and persistence of *Salmonella* in soil. In Field Experiments 2 and 3, *Salmonella* could not be detected by day 10 but our plots were subject to the vicissitudes of weather. In Field Experiment 2, temperatures reached highs of 29°C and in

Field Experiment 3, there was heavy rain in the day following the rainfall event and temperatures dipped to below freezing in the 5 days following the rainfall event. These fluctuations in temperature and precipitation may have impacted the short-term and long-term survival of *Salmonella* in the field.

Similarly, *Salmonella* concentrations on produce decreased in both dry and wet plots. For Field Experiment 2, *Salmonella* concentrations decreased by approximately 1 log CFU per gram in a week. This rate of decay is slightly lower than a previous study of *Salmonella* survival on parsley that observed a 1-3 log CFU/g decay in a week (Kisluk and Yaron 2012). In contrast to our study, Cevallos-Cevallos et al. observed a decrease in concentration of 5.5 log CFU per gram 35 hours after the splash event (Cevallos-Cevallos et al. 2012). In this study, *Salmonella* contamination of produce decreased by less than one log CFU per sample within one hour of contamination. This was likely due to the extremely light rainfall event that occurred following sample collection after the initial rainfall event. This "wash-off effect" was also observed in the study of *Salmonella* splash by Cevallos-Cevallos *et al.* (Cevallos-Cevallos et al. 2012)

Our cross-sectional surveillance of soils in southern Georgia was limited by sampling feasibility but we were able to detect *Salmonella* in the soil of one farm near the location of our field experiments. Capturing a representative soil sample from each farm is difficult and the low prevalence detected may be the result of undersampling. The presence of *Salmonella* in soil in this study is consistent with another study in this region that found *Salmonella* in wet sediment near irrigation ponds (Luo et al. 2015). This study was unable to determine the source of *Salmonella* in the positive sample—no biological soil amendments of animal origin

were applied in the past five years. However, *Salmonella* is regularly detected in the surface water of southern Georgia and surface water is often used for irrigation of produce. This may have resulted in the application of *Salmonella*-contaminated water throughout the field. The soil may also have been contaminated by wildlife intrusion—the managers of the farms in this study reported the prevalence of wildlife in the area.

This combination of the presence of *Salmonella* in the environment and the evidence of splash transfer could present a concern for fresh produce growers seeking to manage the safety of their produce. These results also highlight the potential for produce contamination wherever *Salmonella* (and other pathogens) are detected in soil, where manure is applied to soil, or wildlife feces is found on fields. However, our results on *Salmonella* decay on produce suggest that growers may be able to mitigate the produce safety risk of splash transfer during irrigation and rainfall events by waiting to harvest. In addition, management practices that reduce splash from rain and overhead sprinkler irrigation may be incorporated into production systems. One example of a potential management practice is to leave cover crops on what are now bare soil areas. More information is needed to determine a science-based yet economically viable waiting period and other management practices for growers to implement.

It is important to note that the *Salmonella* concentrations used in this study were likely greater than can be expected in fields following an extended harvest interval and that the inoculum was more diffusely spread through the plot than can be expected in cases of point source contamination from wildlife intrusion. However, the *Salmonella* concentrations used in this study are comparable, if not lower, than what is observed in *Salmonella* shedding for animals,

which in one study, ranged 3-6 log CFU/g in feces of inoculated pigs (Pires et al. 2013). Thus, the splash transfer observed with our experimental conditions could feasibly occur in fields where untreated biological soil amendments of animal origin have previously been applied, fecal contamination from wildlife is present, and contaminated surface water has been applied to soil during irrigation.

Our results demonstrate the potential of splash transfer as a route of preharvest contamination of produce crops, and in particular highlights the impact of antecedent precipitation conditions and soil moisture on the phenomenon of splash contamination. The impact of splash transfer should be incorporated into future produce safety risk assessments. A comprehensive understanding of the drivers of *Salmonella* survival in the environment and the subsequent risks of splash transfer onto produce will inform best practices for preharvest crop management, which are crucial for limiting foodborne pathogen transmission.

Acknowledgments

We would like to thank Mr. Ricky Fletcher, who operated the rainfall simulator and provided the technical guidance for running the simulation experiment, and the student research assistants working with Dr. Timothy Coolong.

Figure 3-1: Overview of the four experiments in this study: Microcosm Experiment, Field Experiment 1, Field Experiment 2, and Field Experiment 3. The size, layout, locations, samples collected, experimental conditions, and outcome measurements for each experiment are indicated.

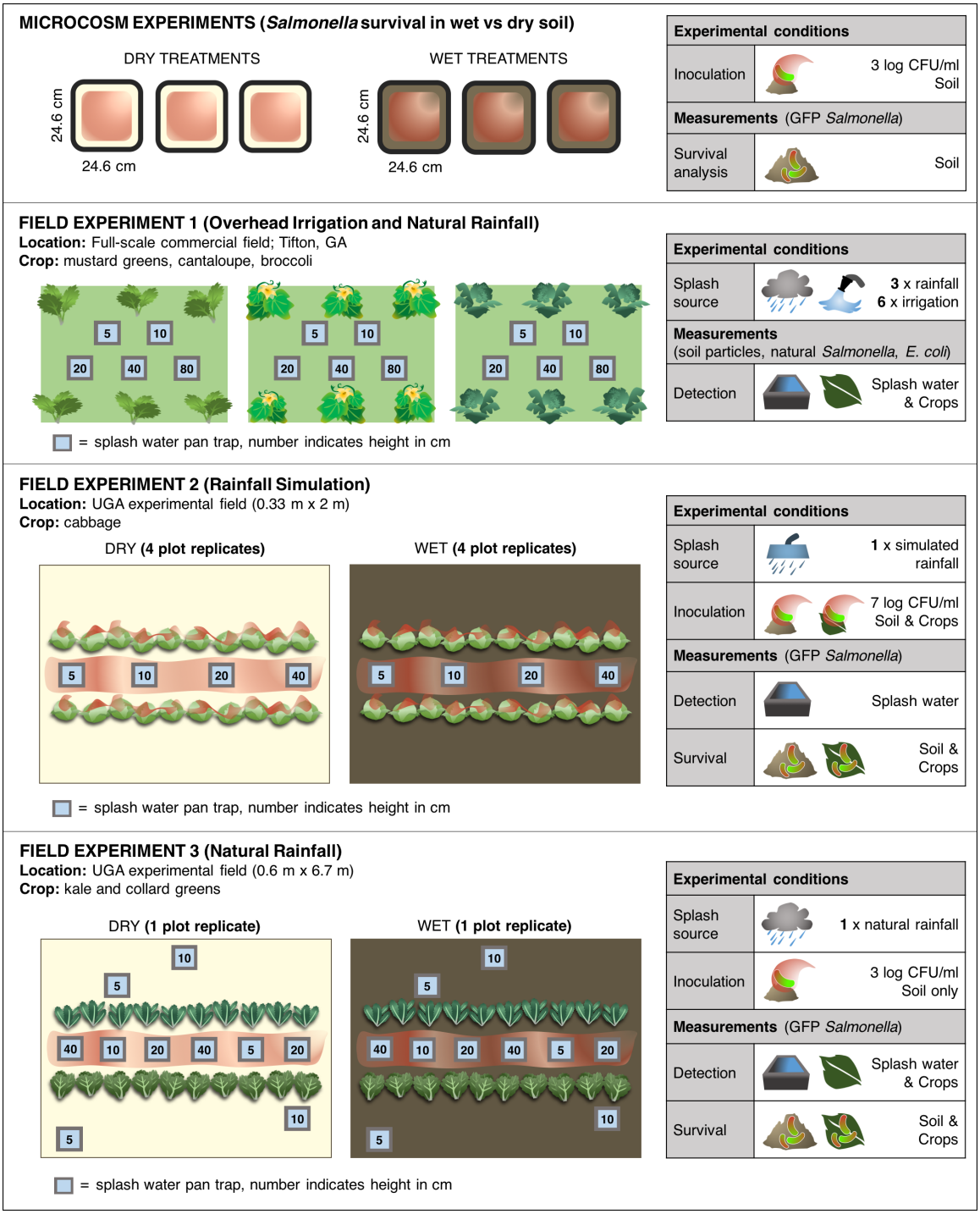


Figure 3-2: Diagram of Field Layout in Field Experiment 1. The project was conducted around one irrigation pond on a large vegetable farm in southern Georgia from 2013-2014. Samples were collected during the growth cycle of three different crops in the fields surrounding the pond. Broccoli and cantaloupe were grown under a center pivot irrigation system and the mustard greens under a solid set irrigation system. Sampling periods for each crop type are indicated.

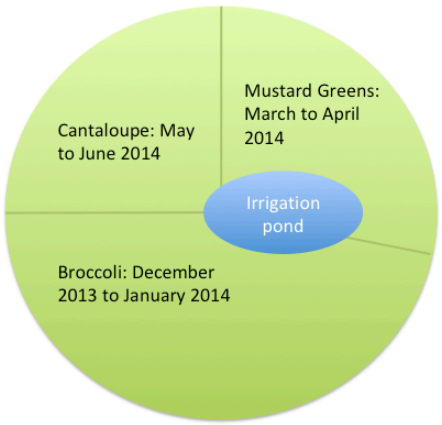


Figure 3-3: Splash Pan Installation in Field Experiment 1. Views of the splash pan installation in the mustard greens field are shown.

Figure 3-4: Map of soil surveillance sampling sites. In October 2017, 105 soil samples were collected from eight farms at University of Georgia research and education centers located across southern Georgia. At each farm, samples were collected to gather a set of samples that represented the entire area of the farm. Research and education center locations are indicated with triangles and labeled with their name.

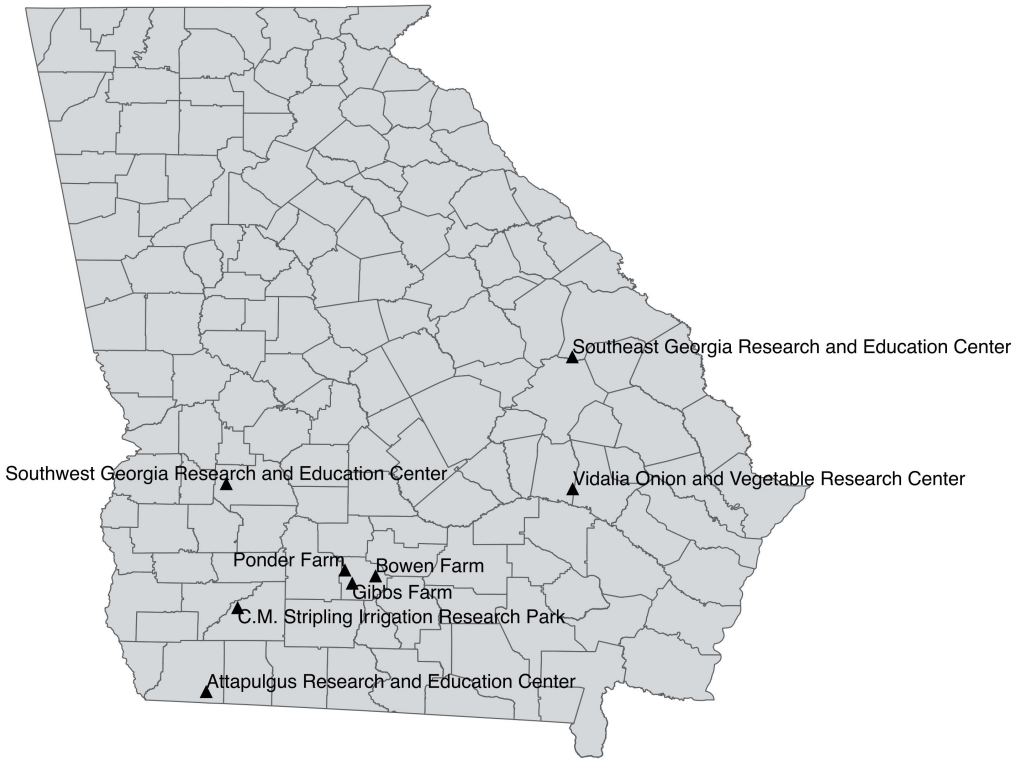
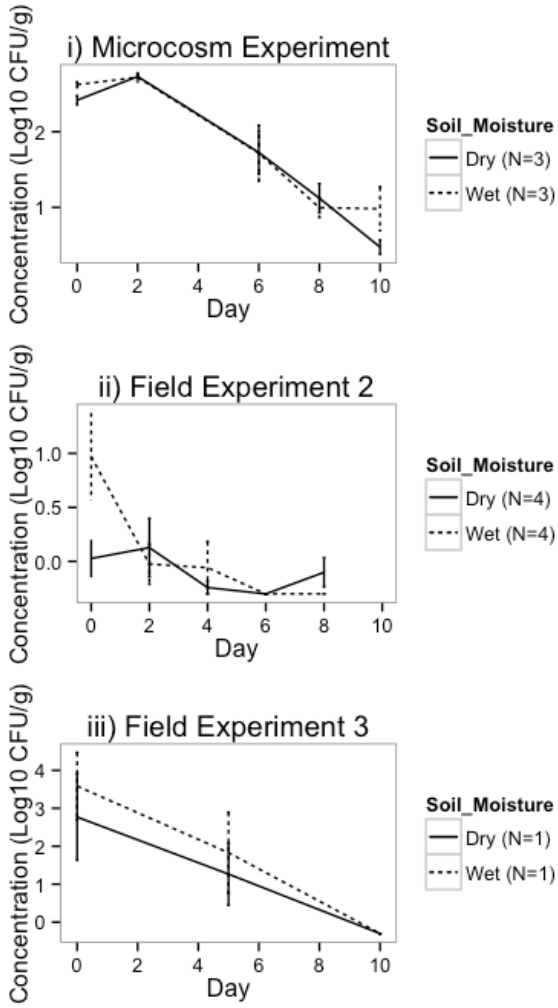


Figure 3-5: *Salmonella* survival in soil and on produce. Comparison of $\log_{10}$ *Salmonella* concentrations (CFU/g) over time by soil moisture content (dry vs. wet) in Microcosm Experiments and Field Experiments 2 and 3. In each graph, the number (N) of microcosms/field plots for each experiment is indicated in the corresponding legend. On each sampling day, a) 3 soil and b) 6 produce samples were collected from each microcosm or plot. Produce survival was only assessed in the field experiments. Error bars represent the standard error of the mean concentrations on each day.

a) Soil



b) Produce

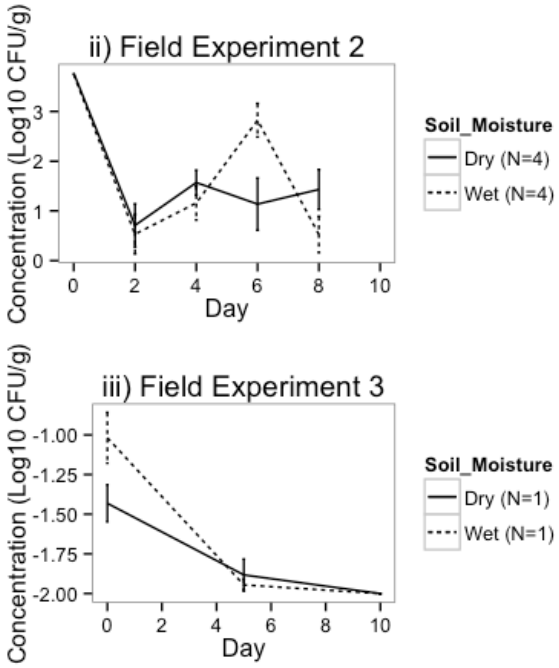


Table 3-1: *Salmonella* die-off rates in soil. Comparison of die-off rate constants (*k*) by soil moisture content in three experimental scenarios. For each experiment, die-off rates were estimated for each soil moisture content category by modeling *Salmonella* concentrations over time. Daily decay was estimated using log-linear regression models based on Chick's Law. Rates are presented in CFU per gram per day (CFU g^{-1} d^{-1}). The p-values for the die-off rates (regression coefficients in log-linear model) are indicated in parentheses.

	Die-off rate constant, *k* (*p*-value[1])		
	Microcosm Experiment	Field Experiment 2	Field Experiment 3
Dry	0.49 (<0.001)	0.08 (0.179)	0.71 (0.023)
Wet	0.45 (<0.001)	0.25 (<0.001)	0.89 (0.008)

[1] *p*-value from t-tests of die-off rate (day^{-1}) regression slopes

Figure 3-6: Transport of soil particles in splash water of Field Experiment 1. A comparison of the concentration (mg/l) of total suspended solids (TSS) in splash pans by height and type of splash-inducing event is shown. Error bars represent the standard error of the mean concentrations at each height. See text for experimental details.

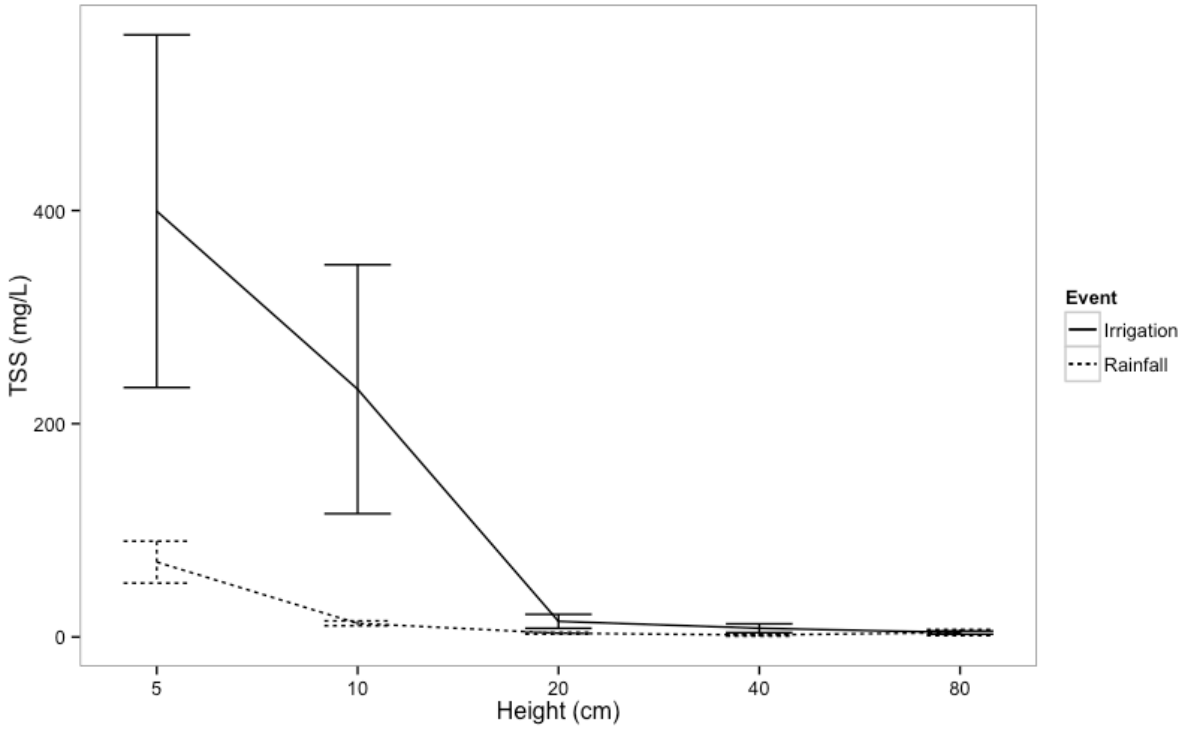
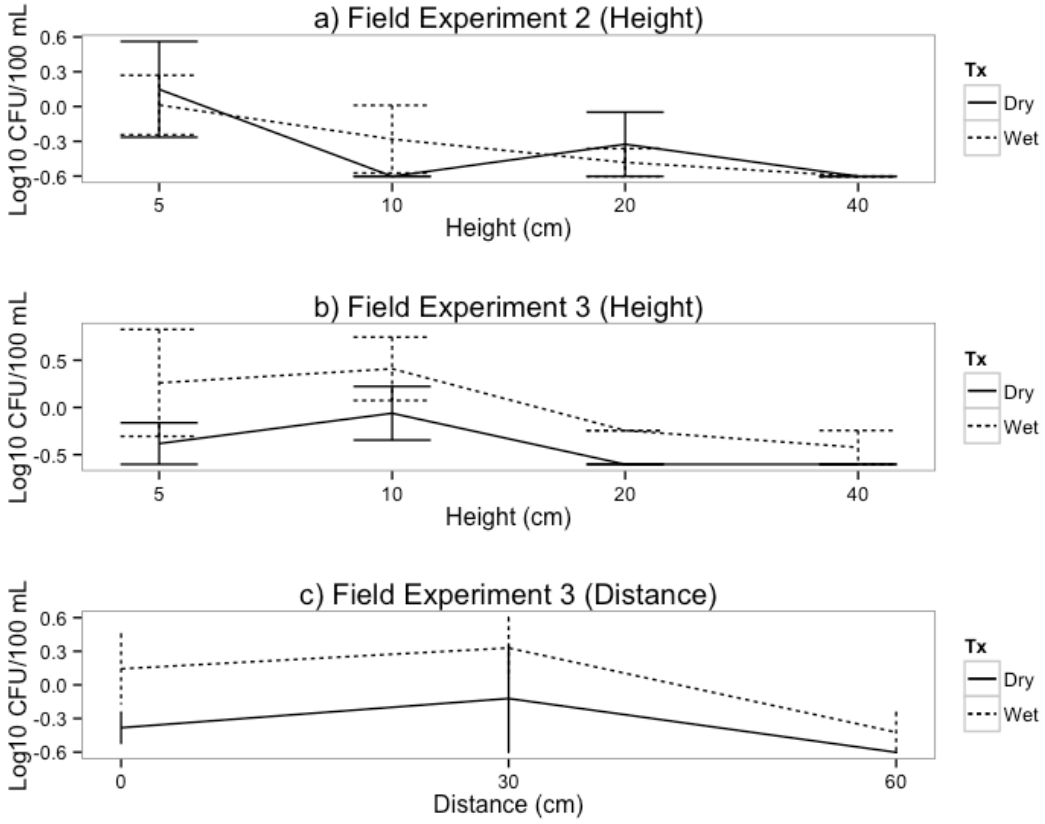


Table 3-2: *Salmonella* concentrations in splash water. For Field Experiments 1, 2, and 3, mean *Salmonella* concentrations (±standard deviation) in water collected from splash pans (CFU/100 ml; MPN/100 ml for Field Experiment 1) are shown for pans installed at various heights (5-80 cm). In Field Experiment 1, splash concentrations are compared by height. For Field Experiments 2 and 3, splash concentrations are compared by height and soil moisture content (dry vs. wet). See text and Figure 1 for experimental details. ND = Not detected.

Splash Pan Height (cm)	Field Experiment 1	Field Experiment 2		Field Experiment 3	
		Dry	Wet	Dry	Wet
5	0.02 ± 0.06 (*N*=9)	2.78 ± 3.47 (*N*=4)	1.67 ± 1.67 (*N*=4)	0.38 ± 0.66 (*N*=3)	7.43 ± 11.89 (*N*=3)
10	0.02 ± 0.03 (*N*=9)	ND (*N*=4)	1.14 ± 1.21 (*N*=4)	1.14 ± 1.15 (*N*=3)	3.99 ± 3.42 (*N*=3)
20	ND (*N*=9)	1.60 ± 2.26 (*N*=4)	0.19 ± 0.33 (*N*=4)	ND (*N*=2)	0.57 ± 0.00 (*N*=2)
40	ND (*N*=9)	ND (*N*=4)	ND (*N*=4)	ND (*N*=2)	0.29 ± 0.40 (*N*=2)
80	0.01 ± 0.02 (*N*=9)	--	--	--	--

Figure 3-7: *Salmonella* in splash water of Field Experiments 2 and 3. Comparisons of mean *Salmonella* concentrations ($\log_{10}$ CFU/100 ml) by soil moisture content (dry vs. wet) in water collected from splash pans are shown for Field Experiments 2 and 3. For Field Experiment 2, splash concentrations are compared by a) height (5-40 cm). For Field Experiment 3, splash concentrations are compared by b) height (5-40 cm) and c) horizontal distance (0-30 cm). Error bars represent the standard error of the mean concentration at each height. See text and Figure 1 for experimental details.



Supplementary Table S3-1 – Gravimetric analyses of soil moisture content. For each experiment, the mean (± standard deviation) soil moisture content for each soil moisture category is listed. Both microcosm and field experiments were watered every two days. Soil moisture between dry and wet treatments in Microcosm Experiments and Field Experiment 2 were significantly different (p <0.05).

Microcosm Experiment		Field Experiment 2		Field Experiment 3	
Dry (n=5)	Wet (n=5)	Dry (n=5)	Wet (n=5)	Dry (n=3)	Wet (n=3)
0.06 ± 0.03	0.16 ± 0.01	0.04 ± 0.03	0.09 ± 0.03	0.07 ± 0.04	0.15 ± 0.06

Supplementary Table S3-2: *Salmonella* survival in soil. Comparison of mean (± standard deviation) $\log_{10}$ *Salmonella* concentrations (CFU/g) over time by soil moisture content in the microcosm- and field-based experiments. Day refers to number of days post-inoculation.

	Microcosm Experiment 1		Field Experiment 2		Field Experiment 3	
Day	Dry (*N*=9)	Wet (*N*=9)	Dry (*N*=12)	Wet (*N*=12)	Dry (*N*=3)	Wet (*N*=3)
0	2.42 ± 0.18	2.62 ± 0.11	0.79 ± 0.26	1.39 ± 0.99	2.77 ± 1.97	3.59 ± 1.53
2	2.73 ± 0.09	2.72 ± 0.17	0.55 ± 0.75	0.33 ± 0.49		
4			0.31 ± 0.09	0.50 ± 0.67		
5					1.45 ± 1.10	1.99 ± 1.57
6	1.73 ± 0.86	1.72 ± 1.11	0.29 ± 0.08	0.32 ± 0.08		
8	1.12 ± 0.58	1.00 ± 0.39	0.43 ± 0.27	0.30 ± 0.12		
10	0.47 ± 0.28	0.98 ± 0.87			-0.52 ± 0.01	-0.45 ± 0.11

Supplementary Table S3-3: *Escherichia coli* concentrations in splash water. For Field Experiment 1, mean *E. coli* concentrations (±standard deviation) in water collected from splash pans (MPN/100 ml) are shown for pans installed at various heights (5-80 cm). In Field Experiment 1, splash concentrations are compared by height and whether the splash event was induced by irrigation or rainfall. See text for experimental details.

Height of Splash Pan (cm)	Irrigation	Rainfall
5	ND	23.40 ± 37.94
	(*N*=6)	(*N*=3)
10	0.40 ± 0.89	2.57 ± 2.29
	(*N*=6)	(*N*=3)
20	2.47 ± 6.04	ND
	(*N*=6)	(*N*=3)
40	9.55 ± 23.39	ND
	(*N*=6)	(*N*=3)
80	0.87 ± 2.12	ND
	(*N*=6)	(*N*=3)

CHAPTER FOUR

Precipitation and Salmonellosis Incidence in Georgia: Interactions Between Antecedent Rainfall Conditions and Extreme Rainfall Events

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Abstract

Compared to other regions of the United States, the southeast consistently has high salmonellosis incidence rates, but the drivers of disease remain unknown. *Salmonella* is regularly detected in the natural environment in this region, leading to exposure opportunities through human interaction with soil, water, plants, and wildlife. Rainfall patterns may impact the survival and transport of *Salmonella* in the environment in ways that can affect disease transmission. This study investigated the impact of extreme rainfall events (above the 90th percentile of daily rainfall levels) and rainfall conditions antecedent to these events (tertiles of eight-week sums of daily rainfall levels) on county-level salmonellosis incidence counts using negative binomial models. In the counties of the Coastal Plain of Georgia, where *Salmonella* is frequently detected in the environment, antecedent rainfall conditions had significant impacts on salmonellosis counts. When considered a lag of one week, compared to periods of moderate rainfall, dry periods were associated with 8% lower incidence of disease (95% CI: 2-13%) while wet periods were associated with 13% increased incidence (95% CI: 6-19%). Extreme rainfall events were associated with 11% increase in salmonellosis risk (95% CI: 5-18%) when they occurred in moderate rainfall or wet periods. These effects were substantially magnified when restricting analyses to salmonellosis cases attributed to serovars commonly isolated from wildlife and the environment (e.g. Javiana). Given the impact of short-term extreme rainfall events and longer-term rainfall conditions on salmonellosis incidence, efforts should be made to inform the public of the risks of interaction with the environment following heavy rainfall events, especially during the rainy season.

Introduction

Every year over 9 million cases of foodborne illness occur in the United States. Non-typhoidal *Salmonella* is estimated to cause 1 million of these cases and is second only to norovirus as the most common foodborne pathogen (Scallan et al. 2011b). The southeastern region of the United States consistently has higher incidence rates of salmonellosis compared to other parts of the country (Centers for Disease Control and Prevention 2016a).

There have been numerous public health initiatives to better understand the epidemiology of foodborne diseases, such as infections from *Salmonella*. The U.S. Centers for Disease Control and Prevention (CDC) has maintained a Foodborne Disease Active Surveillance Network (FoodNet) since 1995. FoodNet represents a long-standing collaboration between the CDC, the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and 10 state health departments. FoodNet conducts rigorous surveillance and promotes behavioral changes to limit the public's contact with foodborne diseases. However, while the U.S. has seen a marked decrease in the incidence of certain foodborne illnesses over the past two decades, this reduction has not been observed for salmonellosis. In fact, salmonellosis incidence has experienced an overall 35% increase since 2001 (Centers for Disease Control and Prevention 2017a)—national incidence was approximately 11 cases per 100,000 population in 2001 but in 2015, it was 15.74 (Centers for Disease Control and Prevention 2016b).

There are over 2,500 serovars of *Salmonella* but human illnesses have been attributed to fewer than 100 serovars (Centers for Disease Control and Prevention 2015). Salmonellae live and reproduce in the gastrointestinal tracts of humans and other animals and are shed through

feces. Direct or indirect contact with contaminated feces can result in infection. Humans can come in contact with the pathogen through fecal matter in or on food and water, and contact with wild and domesticated animals (Labbe 2013). When *Salmonella* is isolated from clinical and food samples during outbreaks, certain serovars are often associated with animal-derived food commodities (Shah et al. 2017) and others, with plant-derived food commodities (Gomba et al. 2016; Reddy et al. 2016). The serovars found on contaminated plant-derived food commodities have also been isolated from non-livestock reservoirs, such as birds, amphibians, water, and soil (Jackson et al. 2013; Micallef et al. 2012; Srikantiah et al. 2004). This may indicate that these serovars are more often associated with environmental reservoirs as opposed to animal-derived food production, and we therefore refer to them here as 'environmental' serovars. In Georgia, many of these serovars are also frequently detected in clinical cases (Maurer et al. 2015). The prevalence of infection with environmental serovars may indicate the potential for human exposure to *Salmonella* in the environment.

Environmental transmission is further supported by the regular detection of *Salmonella* in surface water and other environmental samples in Georgia and neighboring states (Antaki et al. 2016; Haley et al. 2009; Li et al. 2015; Li et al. 2014; Luo et al. 2015; Strawn et al. 2014). Even in non-host environments, such as soil and water, *Salmonella* has been observed to survive and persist for up to 332 days (Islam et al. 2004; Kisluk and Yaron 2012; Maurer et al. 2015; Winfield and Groisman 2003; You et al. 2006) and ultimately be transported through soil, into water resources, and even onto produce crops. The fate and transport of *Salmonella* in the environment can be impacted by various factors, including temperature, soil moisture, nutrients, and microbial competition (Erickson et al. 2014).

Many studies have found that temperature and precipitation can also influence patterns of enteric disease incidence (Carlton et al. 2016; Levy et al. 2016; Stephen and Barnett 2016). In the United States and Canada, waterborne disease outbreaks have been associated with heavy rainfall events (Cann et al. 2013; Curriero et al. 2001; Jiang et al. 2015; Rose et al. 2000). One study assessed the risks of salmonellosis associated with extreme rainfall events in Maryland (Jiang et al. 2015) but this impact has not been explored for Georgia.

Furthermore, while there appears to be a direct relationship between enteric diseases and temperature, influenced primarily by pathogen taxa (Carlton et al. 2016), the influence of rainfall on enteric disease patterns is more non-linear in nature. This may be due to a first flush phenomenon, which occurs when pollutants accumulate in the environment during dry periods and get dislodged en masse during heavy precipitation events (Bach et al. 2010; Lee et al. 2004). During wet periods, environmental pollutants are constantly diluted and transported and thus an extreme precipitation event is less likely to result in the movement of a large bolus of accumulated contaminants. This phenomenon is often studied in the urban storm water context for chemical pollutants, but may also apply to microbial contaminants in both urban and rural settings. Many previous time-series studies of the climatic drivers of enteric disease have used Poisson and negative binomial regression investigate associations between cumulative precipitation levels or the presence of extremely high precipitation levels (Grjibovski et al. 2012; Grjibovski et al. 2013; Hashizume et al. 2007; Singh et al. 2001) on disease counts. Other studies have assessed the association between extreme rainfall events and diarrheal disease (Bush et al. 2014; Jagai et al. 2015). Few studies have explored both extreme rainfall events and cumulative rainfall. One study

used logistic regression to individually assess the impacts of cumulative rainfall and an extreme precipitation event on waterborne outbreaks (Nichols et al. 2009). Of note, a common theme that emerged from a recent literature review on the effects of rainfall on diarrheal diseases is that the effects of heavy rainfall on diarrhea were magnified after dry periods, suggesting that models should incorporate antecedent rainfall conditions (Levy et al. 2016). For example, in a study in Ecuador, heavy rainfall events were associated with elevated rates of diarrhea following dry weather periods, but associated with reduced rates of diarrhea following wet weather periods (Levy et al. 2009). However, Levy et al. looked at the incidence of diarrhea and not the incidence of disease from specific pathogens, such as *Salmonella*.

To examine these phenomena, and to better understand the impact of climatic drivers on salmonellosis incidence patterns in Georgia, we analyzed the effect of precipitation on disease incidence, using a long-term FoodNet dataset for the state. We explored various aspects of rainfall, including overall levels as well as the timing of extreme rainfall events. In particular, we examined the interaction between antecedent rainfall patterns and extreme rainfall events on salmonellosis incidence in Georgia.

Methods

We evaluated a 20-year dataset of salmonellosis cases in each of the 159 counties of Georgia from January 1997 to December 2016, obtained from the Georgia Department of Public Health (GA DPH), a FoodNet site. In this dataset, serotyping was regularly performed on clinical isolates; information on date of symptom onset, county of residence, serotype, whether the case was part of a recognized outbreak, age, gender, race, and ethnicity was

available. Cases identified as associated with outbreaks, based on GA DPH determination, were excluded from the analysis, because the aim of our study was to analyze sporadic cases that could be attributed to environmental sources. We wanted to avoid the influence of cases associated with national or regional foodborne outbreaks, which are often caused by conditions in food production facilities and therefore not affected by rainfall conditions. During the 1997-2016 period, there were 39,540 salmonellosis cases not associated with known outbreaks. While date of symptom onset was available, we aggregated data to weekly disease counts to overcome the low counts of salmonellosis for some counties.

Weather station data were obtained from the 1098 weather stations in Georgia through the National Climatic Data Center (NOAA National Climatic Data Center 2018). Daily maximum temperature and precipitation levels were obtained from each station. We only included weather stations with at least 75% completeness from 1997-2016 of their daily precipitation. Each county was assigned the closest weather station located within 35 km of the center of the most populous city of the county. Our final dataset used data from 116 weather stations to represent the meteorological conditions in the 159 counties.

A negative binomial model with county-specific random intercepts was used to estimate the association between county-level weekly salmonellosis counts and rainfall conditions. Rainfall conditions included the presence of county-specific extreme rainfall events, cumulative rainfall antecedent to extreme rainfall events (hereafter referred to as antecedent conditions), and the interaction between extreme rainfall events and antecedent conditions. These exposures were considered to assess both the overall effects of rainfall, as well as the impact of a first-flush phenomenon on salmonellosis counts in Georgia. Sensitivity analyses

investigated the impacts of lags of one to three weeks between extreme rainfall events and disease.

The model formula is as follows:

$$\log E[Count_{it}] \sim \beta_0 + \beta_1 Extreme_{it} + \beta_2 Antecedent_{it} + \beta_3 Extreme_{it} * Antecedent_{it} + \beta_4 Temperature_{it} + \beta_5 Season_{it} + f(Week\ Number_{it}) + \log(Population_{it})$$

$Count_{it}$ refers to the salmonellosis count in county *i* during week *t*. In this analysis, an extreme precipitation event was defined as a daily precipitation greater than the county-specific 90th percentile (over the 20-year study period). The dichotomous variable, *Extreme*, was a county-specific variable that referred to whether at least one extreme precipitation event occurred in county *i* during the week *t-1*. Antecedent conditions were determined using eight-week sums of daily precipitation levels occurring in the eight weeks prior to analysis, which was similar to another study of precipitation and diarrheal disease (Carlton et al. 2013). Eight-week sums greater than or equal to the county-specific 67th percentile over the 20-year study period were considered high ('wet periods'), sums lower than the county-specific 33rd percentile were considered low ('dry periods'), and the rest were considered intermediate ('moderate rainfall periods'). These antecedent condition categories were also similar to the categories used by Carlton et al (Carlton et al. 2013). These three levels were represented by the categorical variable, *Antecedent*.

In each model, six permutations of rainfall conditions were considered as exposures. Following each of the three eight-week periods of cumulative rainfall (dry/moderate/wet), an extreme rainfall event may or may not have occurred. For each exposure permutation (e.g. wet conditions antecedent to an extreme rainfall event), the associated risk of salmonellosis was estimated.

An offset for population was included in the model to account for differences in county population. Population data were obtained through the Georgia Department of Public Health's Online Analytical Statistical Information System (Georgia Department of Public Health 2018). We controlled for county-specific *Temperature*, using weekly mean temperature; *Season* using a 4-level categorical variable (winter, spring, summer, fall) as categorized by the March equinox, June solstice, September equinox, and December solstice; and long-term trend using natural cubic splines for *week number* in the study, with 20 degrees of freedom for the 20 years of the study. Analyses were conducted using R 3.1.2 (R Core Team 2015).

The Fall Line of Georgia is a boundary line between two large geologic regions of Georgia, separating the Piedmont region from the Coastal Plain region. It runs from Columbus in the west to Augusta in the east (Figure 4-1). The Piedmont, Blue Ridge Mountains, and Ridge and Valley regions lie to the north of the Fall Line while the Upper and Lower Coastal Plain lie to the south. Salmonellosis incidence is higher in the Coastal Plain region and most reports of environmental occurrence of *Salmonella* in Georgia have been reported in this region (Antaki et al. 2016; Haley et al. 2009; Li et al. 2014; Luo et al. 2015; Maurer et al. 2015). Because of the prevalence of *Salmonella* in environmental samples from the Coastal Plain, precipitation patterns can impact pathogen survival and transport in the environment

and in turn, have a greater impact on exposure to *Salmonella* among citizens residing in the Coastal Plain. Therefore, we stratified our analyses by county location relative to the Fall Line. In this paper, the counties south of the Fall Line are considered the 'Coastal Plain' counties and those north of the Fall Line are referred to as the 'Northern' counties.

To investigate the relationship between climatic factors and salmonellosis attributed to serovars more specifically associated with environmental exposure, we modeled county-level weekly salmonellosis counts and restricted our analysis to the non-livestock reservoir serovars discussed by Jackson et al.: Javiana, Litchfield, Mbandaka, Muenchen, Poona, and Senftenberg (Jackson et al. 2013). This negative binomial mixed effects model estimated the same associations as the model with salmonellosis counts from all serovars. To differentiate from the model of all salmonellosis cases in Georgia, we refer to this as the 'environmental serovar' model.

Results

The mean annual incidence throughout the study period in all counties of Georgia was 27 per 100,000 population, with an elevated annual incidence of 39 per 100,000 population in the Coastal Plain counties. A summary of salmonellosis cases in each region (Coastal Plain and Northern) can be found in Figure 4-1 and Table 4-1.

Extreme precipitation events were associated with an 11% increase in risk (IRR: 1.11; 95% CI [1.05, 1.18]) when they occurred following moderate rainfall and wet periods. Extreme precipitation events following dry periods were associated with a 4% increase in risk, but this effect was not statistically significant (IRR: 1.04; 95% CI [0.98, 1.11]). These effects were

only seen in the Coastal Plain counties, and not in the Northern counties (Table 4-2; Supplementary Table S4-1).

In the absence of an extreme precipitation event in the Coastal Plain, compared to moderate rainfall periods, dry periods were associated with an 8% lower salmonellosis risk (IRR: 0.92; 95% CI [0.87, 0.98]), while wet periods were associated with a 13% higher risk (IRR: 1.13; 95% CI [1.06, 1.19]) (Table 4-2; Supplementary Table S4-1).

The effects of precipitation conditions on salmonellosis incidence in the Coastal Plain counties were even more pronounced when limiting the analysis to cases associated with environmental serovars (Table 4-2, Figure 4-2). For all antecedent categories, extreme precipitation events were associated with a significant increase in risk, compared to the reference condition of no extreme event during a moderate rainfall period. The increase in risk associated with an extreme precipitation event ranged from 20% during dry periods (IRR: 1.20; 95% CI [1.06, 1.35]) to 22% in periods with moderate rainfall (IRR: 1.22; 95% CI [1.09, 1.37]) to 34% in wet periods (IRR: 1.34; 95% CI [1.20, 1.49]). In the absence of an extreme precipitation event, wet periods had a 29% higher risk compared to moderate rainfall periods (IRR: 1.29; 95% CI [1.16, 1.44]). Again, these effects were only seen in the Coastal Plain counties, and not in the Northern counties.

When considering lags of two to three weeks, the magnitude of our results decreased. The results of these lagged models can be found in Supplementary Table S4-2.

Discussion

The results of this study indicate a strong, positive association between precipitation variables and salmonellosis incidence in Georgia. In this analysis, we were particularly interested in the timing of heavy rainfall events, and in particular whether antecedent conditions (defined as rainfall in the prior eight weeks) modified the impact of extreme rainfall events on disease incidence. In the Coastal Plain region, extreme rainfall events trended toward increased risk for all levels of antecedent conditions for all serovars, and even more so for the subset of environmentally-associated serovars (Table 4-2). These results highlight the potential impact of wet periods and extreme precipitation events on salmonellosis risk.

One of the strengths of this study was the analysis of salmonellosis incidence by location and serovar. The observed relationships with rainfall in this study were driven primarily by the Coastal Plain region, where *Salmonella* is regularly detected in the environment (Antaki et al. 2016; Harris et al. 2018; Li et al. 2014; Maurer et al. 2015). The presence of amplified risks in the Coastal Plain region lends support to the idea that salmonellosis transmission can occur via environmental exposure routes. We would expect that given the even distribution of risk factors, such as age, poultry consumption, and handwashing behavior, throughout Georgia, incidence would be similar throughout the state; instead, incidence is elevated in the Coastal Plain, where *Salmonella* is regularly detected in the environment.

When analyzing salmonellosis cases attributed to environmental serovars, wet periods had a 29% higher risk compared to moderate rainfall periods and extreme precipitation events were associated with a 20-34% increase in risk. This magnified association between precipitation patterns and salmonellosis from the subset of *Salmonella* serovars that are more

associated with the natural environment (Figure 2), further strengthens the evidence for the importance of rainfall-mediated transmission of *Salmonella* in southern Georgia.

Because the results of this study indicate a strong association between precipitation and disease, an identification of the critical environmental exposure pathways for *Salmonella* infection and the ways in which these pathways are impacted by precipitation is necessary. Also necessary is a better understanding of the mechanisms underlying the relationship between salmonellosis and climatic conditions. Given the greater association between precipitation and environmental serovar infection, wet conditions may have an impact on specific environmental reservoirs. Infections from *Salmonella* Javiana, one of the environmental serovars we considered, are most commonly found in the southeastern region of the United States (Centers for Disease Control and Prevention 2016b, 2017b). It has been shown that Javiana infections are associated with wetland presence in Maryland and Georgia and this may be due to the role of wetlands as a habitat for environmental reservoirs, such as reptiles and amphibians (Huang et al. 2017; Srikantiah et al. 2004). Changes in the frequency and duration of droughts and heavy rainfall have the potential to impact wetland quantity and quality. In turn, they can influence the reproductive success and dispersal of reptiles and amphibians and ultimately, the prevalence of *Salmonella* in the environment (Lind 2008; Walls et al. 2013).

Wet conditions may also promote the survival and transport of *Salmonella* in environmental matrices, such as soil, water, and plants. Soil saturation during wet periods can result in surface water runoff during high rainfall events, which can facilitate the transport of contaminants in the environment (Detty and McGuire 2010; Kibet et al. 2014; Penna et al.

2011). Even when soil is not saturated, the intensity of extreme precipitation events can dislodge contaminants and transport them through soil, into water sources, and potentially onto crops (Barak and Liang 2008; Harris et al. 2018; Islam et al. 2004; Islam et al. 2005; Jacobsen and Bech 2012; Keraita et al. 2007; Park et al. 2012). The increased survival of *Salmonella* during wet conditions and the increased overland flow of *Salmonella* during extreme rainfall events when soil is wet may result in *Salmonella* contamination of surface water and crops. Human exposure to contaminated surface water, either through recreational use or consumption of crops irrigated with surface water, represents one pathway in which rainfall conditions can increase disease risk.

In prior studies of salmonellosis trends by age and sex, while subtle, salmonellosis incidence in males is higher than incidence in females under 20 years, after which age, the trend is inverted (Centers for Disease Control and Prevention 2017b; Reller et al. 2007). This may be due to increased contact with wild and domesticated animals and natural environments in male children compared to female children. It has been posited that the higher incidence in females over 20 may be driven by exposure through fresh produce consumption and food preparation (Boore et al. 2015). Age- and sex-related trends of salmonellosis were not explicitly explored in this analysis but future studies should investigate the behavioral differences that impact salmonellosis incidence.

This study has several strengths. One strength of this study is the geographic distribution of precipitation conditions and salmonellosis cases across all 159 counties of Georgia. Another strength is the twenty-year duration of weather exposures and disease outcomes examined, which considered the impacts of weekly variations in precipitation on weekly case counts. To

our knowledge, this is the first study to assess the impacts of both long-term precipitation and short-term precipitation and the interaction between these two types of precipitation conditions; this study examined antecedent rainfall and extreme rainfall events on salmonellosis. The combined impact of antecedent rainfall conditions and extreme rainfall on salmonellosis incidence in the state of Georgia is consistent with other studies that have shown increase in infections from *Salmonella*, and other enteric pathogens such as *E. coli* O157:H7, *Cryptosporidium*, and *Campylobacter*, following the individual impacts of extreme rainfall (Jiang et al. 2015; Thomas et al. 2006) and rainy periods (Grjibovski et al. 2013; Nichols et al. 2009). Most studies that have assessed the combined impacts of antecedent conditions and extreme rainfall have focused on sediment and contaminant transport in runoff. These studies have similarly found an increase in transport in soil with high moisture content (Callahan et al. 2017; Tallon et al. 2007). Better understanding the impact of precipitation patterns—and not just cumulative precipitation levels or the presence of extreme events—on salmonellosis can help us better predict changes to disease incidence and identify key time points for public health interventions that target the minimization of public exposure to pathogens.

One important caveat to investigating climatic drivers of salmonellosis incidence is the abundance of ways in which people can become exposed to *Salmonella*. This study explores the association between climate and salmonellosis cases but ultimately we cannot definitively know the sources of infection that may be driving incidence. The environmental serovars we considered are commonly associated with plant-based food commodities but are not limited to these commodities and thus, it is difficult to pinpoint produce consumption as the source. We also do not have data to differentiate between exposure from food consumption versus

interaction with the natural environment. There are many behaviors that are influenced by climatic conditions but are also associated with human exposure to *Salmonella*, such as gardening or swimming. We controlled for season to address these types of behaviors. Additionally, we would expect that trends in behaviors increasing exposure would be consistent across the state.

Salmonella infection in this region—even with the environmental serovars—is likely driven by an array of exposure pathways. In spite of the uncertainty surrounding the sources of infection, this study was able to identify that precipitation may play a far greater role in infections with certain serovars than with others. Better understanding the exposure pathways involved in the transmission of these serovars will help identify leverage points to reduce salmonellosis risk.

One major limitation of this study was that we were limited to county-level data, because of the low frequency of weekly disease counts by census tract. To estimate county-level weather data, we used data from the weather station closest to the most populous city in each county, which was up to 35 km away from that city (this represents the maximum). Additionally, even when weather stations were close to the most populous cities, individual salmonellosis cases might have occurred far from these cities. Daily precipitation values can vary widely within a county and thus, some exposure misclassification for the precipitation data may have resulted. We expect that while the presence of an extreme precipitation event in one week may have differed within each county, the categorization of the eight-week antecedent conditions would have been similar throughout the county.

In addition, only cases that were not part of an outbreak were considered for this analysis. However, we may have included some outbreak cases in the analysis if they were not recognized as such by the GA DPH. We expect though that the inclusion of these cases would have been systematic throughout the state and the study period regardless of precipitation patterns and thus, would not have biased our estimates.

It is important to note that estimates of salmonellosis rely on people seeking care and patients and healthcare providers submitting specimen samples. Symptoms of *Salmonella* infections often resolve on their own in four to seven days and many people do not seek medical attention. Those who seek out their healthcare providers are often those with the most severe symptoms (usually young children and the elderly). Moreover, *Salmonella* infections must be laboratory-confirmed and often rely on culture-dependent methods of pathogen detection. Recently, many laboratories have begun to use culture-independent methods with greater sensitivity than the culture-dependent methods. There is some evidence that prior surveillance efforts may have failed to capture many cases of salmonellosis (Huang et al. 2016; Langley et al. 2015; Marder et al. 2017). Self-reporting biases and laboratory method sensitivity may have led to an underestimation of disease incidence. However, our reliance on reports from healthcare providers and laboratory confirmations imposed a systematic error throughout Georgia counties and would not have biased the risk estimates in either direction. While greater care-seeking behavior may be observed for the young and the elderly due to the susceptibility of these populations, the age-distributions in each county were similar and thus, even with the overrepresentation of these age groups in the surveillance data, we do not believe this overrepresentation introduced bias to the study.

Given the contribution of climatic factors to salmonellosis risk, better understanding of the impact of climate on environmental pathogen transmission is critical for planning adaptation measures for potential changes in climate. Our results suggest that extreme rainfall events and periods of prolonged wetness or dryness can impact salmonellosis risk. Potential increases in periods of drought (Dai 2012; Trenberth 2011) may be protective against exposure and infection but the increased frequency and intensity of extreme precipitation events (Fischer and Knutti 2016; Kunkel et al. 2013; Prein et al. 2016) may increase salmonellosis risk. Under a high emissions scenario, which assumes continued increases in emissions, southern Georgia is expected to experience a 15-20% increase in the number of days when precipitation is greater than one inch by 2041-2070 (compared to 1980-2000) (Kunkel et al. 2013), which is similar to the threshold for extreme precipitation event in our study. This suggests that in this high emissions scenario, there may be an elevated risk of salmonellosis in southern Georgia. A comprehensive understanding of these climatic changes and the exposure pathways they influence will help improve public health measures to mitigate salmonellosis risk.

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Figure 4-1: Average salmonellosis incidence (per 100,000 population) in Georgia by county during 1997-2016. Incidence was estimated using cases as reported in the FoodNet system (data courtesy of the Georgia Department of Public Health). Counties of the Coastal Plain are outlined in light gray. These counties occur south of the Fall Line that separates the major geologic regions of the state. The top four most populous cities of Georgia (Atlanta, Columbus, Augusta, Savannah) are indicated.

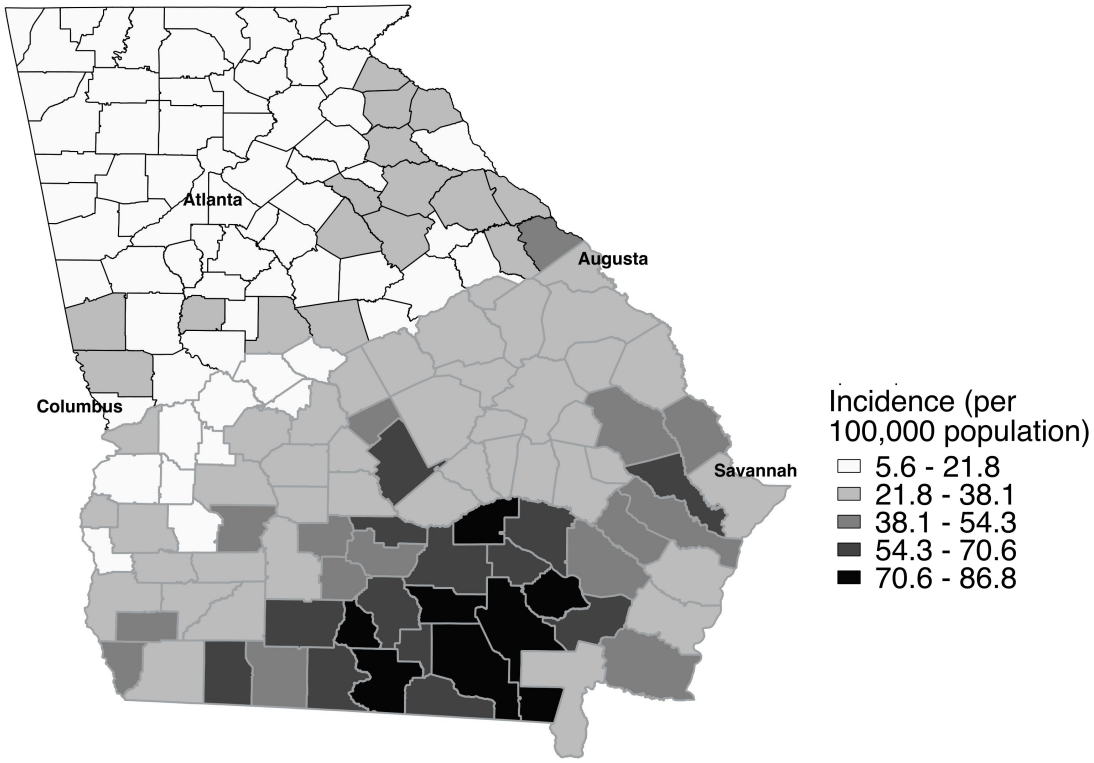


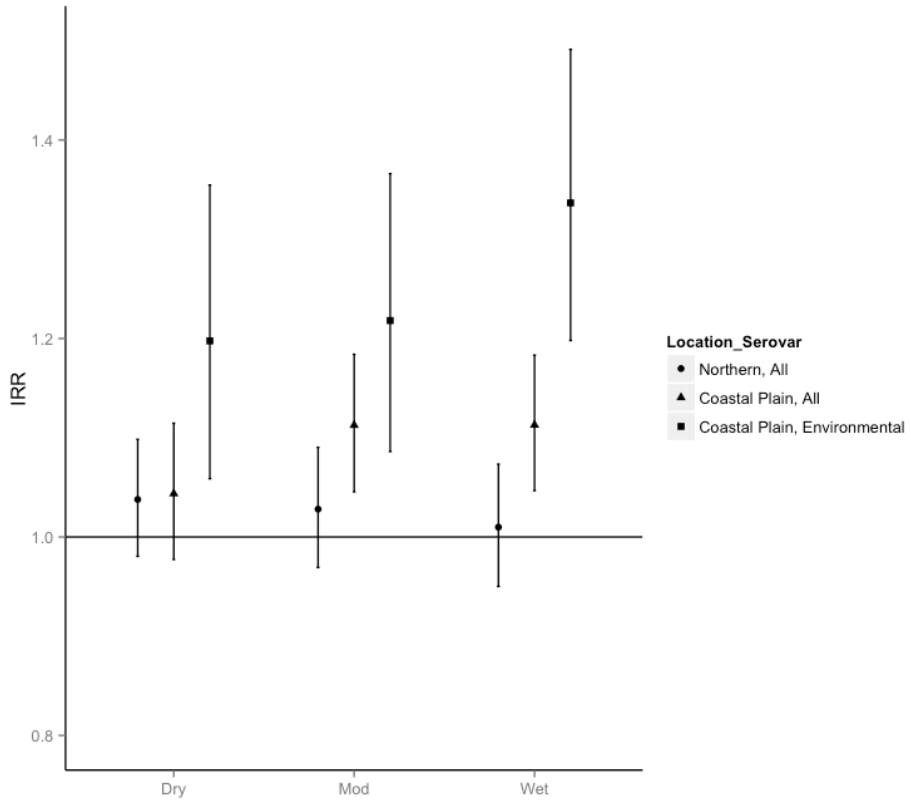
Table 4-1: Comparison of salmonellosis counts in Coastal Plain and Northern counties during 1997-2016. The total number of cases over the study period is displayed for each region of Georgia. Salmonellosis counts are stratified by serovar type (all serovars or environmental serovars, see text for distinction), season of disease onset, and patient demographics, such as gender, age, and race. For race, the four largest race categories in Georgia were considered. Population refers to the mean yearly population.

		Coastal Plain	Northern
Population		2,938,517	6,197,440
Serovar			
	All	18,982	20,558
	Environmental	5,038	2,643
Season			
	Winter	1,765	2,712
	Spring	2,808	4,398
	Summer	8,263	8,385
	Fall	6,146	5,063
Gender			
	Female	9,429	10,203
	Male	9,459	10,228
Age			
	<1	4,405	3,098
	1-9	6,111	6,374
	10-19	968	1,610
	20-39	1,777	2,917
	40-59	2,266	3,025
	60-79	2,004	1,824
	80+	467	458
Race			
	American Indian/Alaska Native	25	25
	Asian	77	456
	Black or African American	4,129	4,289
	White	11,395	12,143

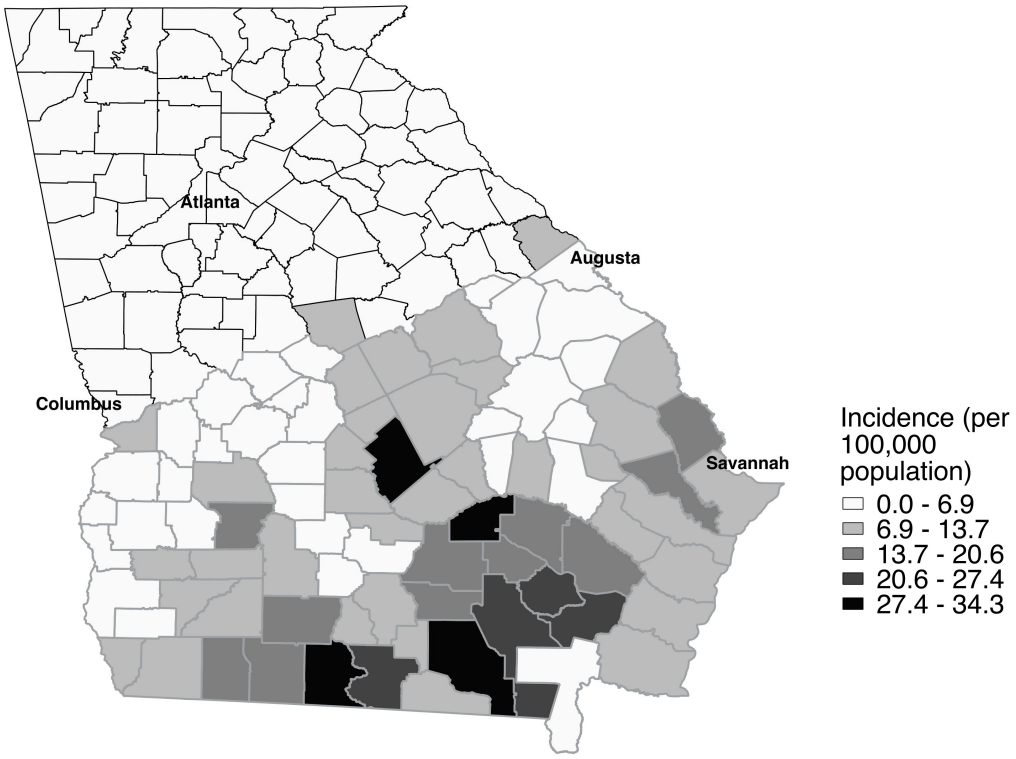
Table 4-2: Incidence rate ratios (IRR) of precipitation-related variables in multivariate models of salmonellosis incidence from all serovars (top) and environmental serovars (bottom). Extreme refers to the presence of an extreme precipitation event at the 90th percentile of daily precipitation levels in the week preceding the week of disease incidence (1-week lag). The presence of an extreme event is indicated as no or yes. Antecedent refers to the antecedent conditions preceding the week in question (dry, moderate rainfall, or wet periods), corresponding to tertiles of total daily precipitation over the prior 8 weeks. IRRs and 95% confidence intervals (CI) for six possible combinations of precipitation conditions in each model are shown. Significant IRRs are in bold.

Extreme	Antecedent	All Counties	95% CI	Coastal Plain Counties	95% CI	Northern Counties	95% CI
All Serovars							
No	Dry	**0.94**	**(0.90, 0.98)**	**0.92**	**(0.87, 0.98)**	1.01	(0.96, 1.07)
	Mod	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
	Wet	**1.08**	**(1.03, 1.13)**	**1.13**	**(1.06, 1.19)**	0.94	(0.89, 1.00)
Yes	Dry	1.02	(0.97, 1.06)	1.04	(0.98, 1.11)	1.04	(0.98, 1.10)
	Mod	**1.09**	**(1.04, 1.13)**	**1.11**	**(1.05, 1.18)**	1.03	(0.97, 1.09)
	Wet	**1.09**	**(1.05, 1.14)**	**1.11**	**(1.05, 1.18)**	1.01	(0.95, 1.07)
Environmental Serovars							
No	Dry	0.97	(0.88, 1.06)	0.96	(0.86, 1.09)	0.95	(0.81, 1.11)
	Mod	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
	Wet	**1.19**	**(1.09, 1.31)**	**1.29**	**(1.16, 1.44)**	0.90	(0.76, 1.06)
Yes	Dry	**1.12**	**(1.01, 1.23)**	**1.20**	**(1.06, 1.35)**	1.05	(0.90, 1.22)
	Mod	**1.18**	**(1.07, 1.29)**	**1.22**	**(1.09, 1.37)**	1.06	(0.91, 1.24)
	Wet	**1.28**	**(1.17, 1.40)**	**1.34**	**(1.20, 1.49)**	1.14	(0.97, 1.34)

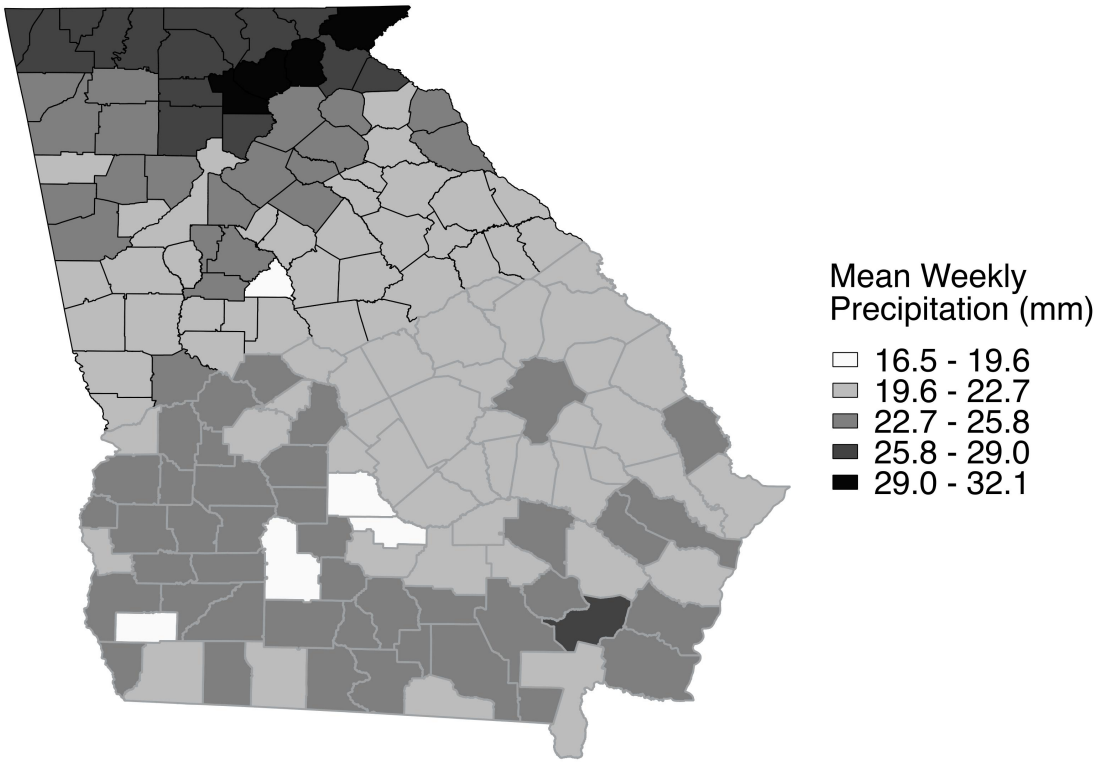
Figure 4-2: Comparison of incidence rate ratios (IRRs) for the impact of an extreme precipitation event (1-week lag) preceded by differing antecedent precipitation conditions by county location and serovar type. Three levels of precipitation conditions in an eight-week period antecedent to an extreme precipitation event (90^{th} percentile of daily precipitation) are displayed, and indicated as dry, moderate (mod), and wet. For each antecedent condition category, the IRRs (and 95% CI) depending on the county location (Northern vs. Coastal Plain) and type of serovar (all serovars vs. environmental serovars, see text for distinction) isolated from cases are shown.



Supplementary Figure S4-1: Average salmonellosis incidence (per 100,000 population) from environmental serovars in Georgia by county during 1997-2016. Incidence was estimated using cases as reported in the FoodNet system (obtained from the Georgia Department of Public Health). Counties of the Coastal Plain are outlined in light gray. These counties occur south of the Fall Line that separates the major geologic regions of the state. The top four most populous cities of Georgia (Atlanta, Columbus, Augusta, Savannah) are indicated.



Supplementary Figure S4-2: Average weekly precipitation (mm) by county during study period according to each county's assigned weather station. Each county was assigned a weather station that fit two criteria: the station was within 35 km of the county's most populous city and had at least 75% completeness in precipitation data. Counties of the Coastal Plain (counties occurring south of the Fall Line separating the major geologic regions of the state) are outlined in gray.



Supplementary Table S4-1: Incidence rate ratios (IRR) of parameters in multivariate models of salmonellosis incidence attributed to all serovars. The dichotomous 'Extreme' variable refers to the presence of an extreme precipitation event (0 = no extreme event, 1 = extreme event) in the week preceding the week of disease incidence. Antecedent ('Ante') precipitation levels in the eight weeks prior to an extreme event were categorized into tertiles of overall precipitation (dry, moderate rainfall, and wet), with moderate rainfall as the reference category. The continuous 'Temperature' variable indicates a ten-degree change in average temperature (degrees Celsius). The categorical variable to control for season (winter, spring, summer, fall) uses winter as the reference category. The parameter estimates for natural splines are not shown here but were included in the model. Significant results are indicated in bold.

	All Counties	95% CI	Coastal Plain Counties	95% CI	Northern Counties	95% CI
Extreme	**1.09**	**(1.04, 1.13)**	**1.11**	**(1.05, 1.18)**	1.03	(0.97, 1.09)
Dry (Ante)	**0.94**	**(0.90, 0.98)**	**0.92**	**(0.87, 0.98)**	1.01	(0.96, 1.07)
Wet (Ante)	**1.08**	**(1.03, 1.12)**	**1.13**	**(1.06, 1.19)**	0.94	(0.89, 1.00)
Temperature	**1.52**	**(1.47, 1.57)**	**1.75**	**(1.66, 1.84)**	**1.37**	**(1.31, 1.43)**
Spring	1.05	(1.00, 1.11)	0.94	(0.86, 1.02)	**1.16**	**(1.08, 1.25)**
Summer	**1.92**	**(1.80, 2.05)**	**2.04**	**(1.85, 2.26)**	**1.77**	**(1.61, 1.93)**
Fall	**1.91**	**(1.82, 2.01)**	**2.42**	**(2.25, 2.61)**	**1.46**	**(1.37, 1.56)**
Extreme*Dry	1.00	(0.94, 1.06)	1.02	(0.93, 1.11)	1.00	(0.92, 1.08)
Extreme*Wet	0.94	(0.88, 1.00)	**0.89**	**(0.82, 0.97)**	1.04	(0.96, 1.13)

Supplementary Table S4-2: Comparison of incidence rate ratios of 1-3 week lags in precipitation-related variables in multivariate models of salmonellosis incidence from all serovars (top) and environmental serovars (bottom) in the Coastal Plain counties. Incidence rate ratios and 95% confidence intervals (CI) for the six possible combinations of precipitation conditions are shown. The presence of an extreme event is indicated as no or yes. The antecedent condition levels are: dry, moderate (mod), and wet. Significant IRRs are in bold.

Extreme	Antecedent	1-week lag	95% CI	2-week lag	95% CI	3-week lag	95% CI
All Serovars							
No	Dry	**0.92**	**(0.87, 0.98)**	**0.92**	**(0.87, 0.98)**	**0.93**	**(0.87, 0.99)**
	Mod	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
	Wet	**1.13**	**(1.06, 1.19)**	**1.07**	**(1.01, 1.14)**	**1.10**	**(1.03, 1.16)**
Yes	Dry	1.04	(0.98, 1.11)	0.99	(0.93, 1.06)	1.02	(0.96, 1.09)
	Mod	**1.11**	**(1.05, 1.18)**	**1.07**	**(1.01, 1.14)**	**1.07**	**(1.01, 1.14)**
	Wet	**1.11**	**(1.05, 1.18)**	**1.13**	**(1.06, 1.20)**	**1.12**	**(1.06, 1.19)**
Environmental Serovars							
No	Dry	0.97	(0.88, 1.06)	0.92	(0.81, 1.03)	0.92	(0.82, 1.04)
	Mod	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
	Wet	**1.19**	**(1.09, 1.31)**	**1.14**	**(1.03, 1.27)**	**1.20**	**(1.07, 1.33)**
Yes	Dry	**1.12**	**(1.01, 1.23)**	0.96	(0.85, 1.09)	1.02	(0.90, 1.16)
	Mod	**1.18**	**(1.07, 1.29)**	1.07	(0.96, 1.20)	**1.13**	**(1.01, 1.26)**
	Wet	**1.28**	**(1.17, 1.40)**	**1.22**	**(1.10, 1.36)**	**1.24**	**(1.11, 1.38)**

CONCLUSION

This dissertation investigated several pathways of human exposure to *Salmonella* in Georgia through myriad lenses. I conducted laboratory-, field-, and population-based studies to examine factors that have the potential to impact salmonellosis risk.

In the first three chapters, I focused on human exposure to *Salmonella* through fresh produce consumption. In Chapter One, I showed that surface water ponds that are used for the irrigation of fresh produce crops in southern Georgia were regularly contaminated with *Salmonella* and *E. coli*. I compared two shoreline-sampling strategies to provide growers an alternative to sampling at the end of the intake pipe in the irrigation ponds. However, there was wide variability in water quality at different areas of the irrigation ponds and the shoreline sampling results did not often agree with the intake sampling results. Furthermore, the spatial heterogeneity in water quality at irrigation ponds suggested the difficulty of collecting a sample that could adequately represent the overall quality of water at the irrigation pond. Because of this, growers should sample water from valves installed at various intervals within the irrigation line, which would provide a better approximation of the quality of water applied to crops. Moreover, even though *Salmonella* was detected in surface water, it was unclear whether this contamination could subsequently be detected on fresh produce. This uncertainty formed the basis for the research objectives outlined in Chapter Two.

In Chapter Two, I determined that *Salmonella* was not only present in surface water irrigation ponds of commercial farms but also in irrigation distribution systems and on fresh produce.

These results suggested that contaminants in surface water might be transported from ponds, into irrigation systems, and onto produce. However, because one *Salmonella* subtype was not consistently found in source water, irrigation systems, and on produce from the same farms, it was difficult to link source water contamination to produce contamination. However, increased sampling efforts may have been able to uncover the same subtype throughout the path irrigation water takes from its source to produce. Additionally, even though we were unable to link surface water contamination to produce contamination through the detection of a common subtype, *Salmonella* was detected on produce from commercial farms, which indicates the potential for produce contamination on these fields. The detection of *Salmonella* on commercial produce—albeit at low prevalence and concentrations—highlight the need for identifying the sources and pathways of contamination. We detected a high level of genetic diversity in *Salmonella* isolates from the study region, which suggested that *Salmonella* contamination on produce farms came from diffuse environmental sources. Thus, minimizing the risk of produce contamination will require a multifaceted approach that targets numerous pathways of contamination, including the use of surface water for irrigation. In addition to protecting surface water irrigation ponds through the construction of riparian buffers to limit runoff or fencing to restrict animal access, growers can cease irrigation close to the commercial harvest so that sufficient die-off can occur. Given the low concentrations of *Salmonella* we detected on crops (approximately 1 MPN on 8 cucumbers or 2 cantaloupes) and the evidence in the literature that *Salmonella* on crops can decay ~2 log CFU/g in the first 48 hours (Kisluk et al. 2012), irrigation cessation several days before harvest may be sufficient. Future research characterizing the rate of decay in the field is necessary to provide growers with a science-based recommendation for a harvest interval.

This study used three different subtyping methods to assess *Salmonella* diversity: serotyping, pulsed-field gel electrophoresis (PFGE), and whole-genome sequencing (WGS). Whole genome sequencing allows for greater discrimination between isolates—in the current study, two isolates with the same PFGE pattern had hundreds of single nucleotide polymorphism (SNP) differences. The current gold standard for *Salmonella* subtyping in epidemiological investigations is PFGE. However, the growing use and affordability of WGS indicate the potential for future outbreak investigations to harness this powerful tool for the efficient identification of disease clusters and traceback investigations.

In Chapter Three, I found evidence of *Salmonella* in soil on farms in southern Georgia and showed that irrigation and rainfall events can result in the splash transfer of *Salmonella* from soil to crops. Furthermore, the field experiments demonstrated that splash transport of *Salmonella* was increased when soil moisture content was high. In Chapter Two, *Salmonella* was detected on cantaloupe and cucumber samples and the source of contamination could not be traced back to the surface water irrigation pond using *Salmonella* subtyping. The evidence of *Salmonella* in soil in southern Georgia and splash transfer in Chapter Three may point to the role of soil splash in produce contamination in our study region. It is important to note that while *Salmonella* could be detected in splash water and also on crops after splash-inducing events, concentrations were low. In Field Experiment 3 of Chapter Three, *Salmonella* concentrations decreased further within one hour of the rainfall event and continued to decrease in the days following the rainfall event. These results indicate that even though *Salmonella* can be transferred onto crops during irrigation and rainfall events, *Salmonella* die-off occurs on crop surfaces. The decay rate we observed might also have been

augmented in the warmer months—our study occurred in the cooler, autumn months of Georgia that are more favorable to *Salmonella* survival (Holley et al. 2006; Semenov et al. 2007; Underthun et al. 2018). Thus, our results indicate that growers may be able to implement a waiting period between irrigation and rainfall events and harvest to account for pathogen die-off. However, through the presence of ambient conditions amenable to *Salmonella* survival, the entry into a viable but non-culturable state, or the formation of biofilms, *Salmonella* can persist and even grow in the field (Ávila-Quezada et al. 2010; Islam et al. 2005; Waldner et al. 2012; Winfield and Groisman 2003). Thus, waiting periods may need to be tailored to field conditions and future research should investigate optimal waiting periods under various scenarios. Another strategy for growers to mitigate risks from splash transfer is the use of cover crops, which can reduce rainfall kinetic energy and soil erosion and in turn, reduce the potential for soil splash (Ma et al. 2014; Ntahimpera et al. 1998; Parlak and Parlak 2010).

The findings from these three chapters highlight the potential risk of *Salmonella* contamination of produce through soil and irrigation water. Moreover, the risk of *Salmonella* transport through splash is exacerbated with higher soil moisture content. This indicates that precipitation conditions resulting in increased soil moisture can modify the effect of subsequent extreme rainfall events on *Salmonella* transport in the environment. This interaction between antecedent rainfall conditions and extreme rainfall events was then explored at the population level in Chapter Four. In Chapter Four, I shed light on the associations between precipitation patterns and salmonellosis risk in Georgia. I found that extreme precipitation events and wet periods are associated with increased risk of disease. The impact of precipitation patterns was greater in the Coastal Plain region of Georgia and

even more enhanced when examining infections from serovars associated with environmental reservoirs of *Salmonella*. These results suggest that environmental exposure may be an important factor in salmonellosis risk in southern Georgia and underscore the value of further elucidating the myriad modes and pathways of *Salmonella* transmission in the environment. The importance of environmental exposures in *Salmonella* infection in Georgia indicates that we may need to warn the public about behaviors and activities that are especially risky following specific meteorological conditions. Many surfers are already aware of the human health risks of recreation following rainfall events and thus, it may be beneficial for public health officials to more widely disseminate warnings about post-rainfall recreational water use and gardening.

I conclude that *Salmonella* presence in the produce production environment, either in surface water or soil, poses a risk for *Salmonella* contamination of crops. The risks to produce safety increase with precipitation and antecedent soil moisture content, which can aid the transport of *Salmonella* into surface water and onto crops. In a similar vein, salmonellosis risk in this region is associated with extreme precipitation events and antecedent precipitation conditions.

These risks to produce safety and public health are costly on many levels. In 2013, the total economic burden of *Salmonella* infections was $3.7 billion, making it the most economically burdensome foodborne pathogen in the United States (Hoffman et al. 2015). This estimate incorporated medical costs, productivity loss, and willingness to pay to reduce risks of death. Additionally, as evidenced by the 2006 outbreak, food safety shocks have the potential to dramatically change consumer behavior and upend the produce industry.

Many of the growers in southern Georgia are committed to produce safety and engage in Good Agricultural Practices in order to minimize the microbial contamination of their fresh fruits and vegetables. However, many of the modes of contamination in the field (e.g. wildlife intrusion and rainfall splash) are difficult to control and thus, zero-risk of preharvest contamination is elusive, if not impossible. This does not mean that produce safety efforts are futile; it means that we need a more comprehensive understanding of the contamination pathways implicated and their relative contributions to foodborne disease risk. This will provide the foundation and knowledge for conducting risk assessments that can inform food safety policy. Moreover, better understanding the contamination pathways can help elucidate key leverage points to minimize the microbial risks of fresh produce consumption. Preventing preharvest contamination likely necessitates a combination of on-farm strategies

There are many reasons to be optimistic about the potential for reductions in foodborne illnesses. As previously mentioned, the increasing accessibility and affordability of whole genome sequencing (WGS) and the rising adoption of culture-independent diagnostic tools will allow us to identify outbreaks faster and provide us the discriminatory power to efficiently conduct trace-back investigations. The results of Chapter Two indicated that whole-genome sequencing has the potential to distinguish variants of the same pulsed-field gel electrophoresis (PFGE) pattern. Similarly, shotgun metagenomics were recently employed to differentiate between two outbreaks in the same year that were attributed to *Salmonella* Heidelberg and had indistinguishable PFGE patterns (Huang et al. 2017).

The increasing global food demand and globalization of food distribution pose a serious challenge to food safety (Quested et al. 2010; Tauxe et al. 2010). In response, we need a full examination of factors that drive contamination in the farm-to-fork continuum. Preharvest contamination is only one of the many parts of the food supply chain that can be targeted but preventing contamination at the preharvest level represents the opportunity to curb contamination early on in the chain. As demonstrated by my dissertation research and other studies of produce safety, the drivers of preharvest contamination are also vulnerable to potential changes in climate (Jung et al. 2014; Liu et al. 2013; Semenza et al. 2012). It is a public health imperative to understand the ways in which food safety will be impacted by changes in climate. Better understanding the pathways of preharvest contamination will help inform the development of interventions to minimize produce contamination and ultimately, help generate climate resilience in our changing food system.

REFERENCES

Abu-Ashour J, Lee H. 2000. Transport of bacteria on sloping soil surfaces by runoff. Environmental Toxicology 15:149-153.

Ahmed W, Goonetilleke A and Gardner T. Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in southeast Queensland, Australia. Can J Microbiol 2010; 56:471.

Angiuoli SV, Salzberg SL. 2011. Mugsy: fast multiple alignment of closely related whole genomes. Bioinformatics 27:334-342.

Antaki EM, Vellidis G, Harris C, Aminabadi P, Levy K and Jay-Russell MT. Low Concentration of *Salmonella enterica* and Generic *Escherichia coli* in Farm Ponds and Irrigation Distribution Systems Used for Mixed Produce Production in Southern Georgia. Foodborne Pathog Dis 2016; 13:551-558.

Arnade C, Calvin L, Kuchler F. 2009. Consumer Response to a Food Safety Shock: The 2006 Food-Borne Illness Outbreak of *E. coli* O157: H7 Linked to Spinach. Applied Economic Perspectives and Policy 31:734-750.

Assouline S. 2004. Rainfall-induced soil surface sealing: A critical review of observations, conceptual models, and solutions. Vadose Zone J 3:570-591.

Atwill ER, Chase JA, Oryang D, Bond RF, Koike ST, Cahn MD, et al. 2015. Transfer of Escherichia coli O157:H7 from Simulated Wildlife Scat onto Romaine Lettuce during Foliar Irrigation. J Food Prot 78:240-247.

Ávila-Quezada G, Sánchez E, Gardea-Béjar AA, Acedo-Félix E. 2010. Salmonella spp. and Escherichia coli: survival and growth in plant tissue. New Zealand Journal of Crop and Horticultural Science 38:47-55.

Bach PM, McCarthy DT, Deletic A. 2010. Redefining the stormwater first flush phenomenon. Water Res 44:2487-2498.

Barak JD, Liang AS. 2008. Role of Soil, Crop Debris, and a Plant Pathogen in *Salmonella enterica* Contamination of Tomato Plants. PLoS One 3:5.

Bartz FE, Lickness JS, Heredia N, Fabiszewski de Aceituno A, Newman KL, Hodge DW, et al. 2017. Contamination of Fresh Produce by Microbial Indicators on Farms and in Packing Facilities: Elucidation of Environmental Routes. Applied and Environmental Microbiology 83.

Behravesh CB, Mody RK, Jungk J, Gaul L, Redd JT, Chen S, et al. 2011. 2008 Outbreak of *Salmonella* Saintpaul Infections Associated with Raw Produce. New England Journal of Medicine 364:918-927.

Benjamin L, Atwill ER, Jay-Russell M, Cooley M, Carychao D, Gorski L and Mandrell RE. 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. International Journal of Food Microbiology 165:65-76.

Beuchat LR. 2006. Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. British Food Journal 108:38-53.

Blaustein RA, Shelton DR, Van Kessel JAS, Karns JS, Stocker MD, Pachepsky YA. 2015. Irrigation waters and pipe-based biofilms as sources for antibiotic-resistant bacteria. Environmental Monitoring and Assessment 188:56.

Boore AL, Hoekstra RM, Iwamoto M, Fields PI, Bishop RD, Swerdlow DL. 2015. *Salmonella enterica* Infections in the United States and Assessment of Coefficients of Variation:

A Novel Approach to Identify Epidemiologic Characteristics of Individual Serotypes, 1996–2011. PLoS One 10:e0145416.

Bowen A, Fry A, Richards G, Beauchat L. 2006. Infections associated with cantaloupe consumption: a public health concern. Epidemiol Infect 134:675-685.

Boyer DG. 2008. Fecal coliform dispersal by rain splash on slopes. Agricultural and Forest Meteorology 148:1395-1400.

Bradford SA, Morales VL, Zhang W, Harvey RW, Packman AI, Mohanram A, et al. 2013. Transport and Fate of Microbial Pathogens in Agricultural Settings. Critical Reviews in Environmental Science and Technology 43:775-893.

Brennan FP, Moynihan E, Griffiths BS, Hillier S, Owen J, Pendlowski H, et al. 2014. Clay mineral type effect on bacterial enteropathogen survival in soil. Science of The Total Environment 468–469:302-305.

Bush KF, O'Neill MS, Li S, Mukherjee B, Hu H, Ghosh S, et al. 2014. Associations between Extreme Precipitation and Gastrointestinal-Related Hospital Admissions in Chennai, India. Environ Health Perspect 122:249-254.

Callahan MT, Micallef SA, Buchanan RL. 2017. Soil Type, Soil Moisture, and Field Slope Influence the Horizontal Movement of *Salmonella enterica* and *Citrobacter freundii* from Floodwater through Soil. J Food Prot 80:189-197.

Cann KF, Thomas DR, Salmon RL, Wyn-Jones AP, Kay D. 2013. Extreme water-related weather events and waterborne disease. Epidemiol Infect 141:671-686.

Carlton EJ, Eisenberg JNS, Goldstick J, Cevallos W, Trostle J, Levy K. 2014. Heavy Rainfall Events and Diarrhea Incidence: The Role of Social and Environmental Factors. Am J Epidemiol 179:344-352.

Carlton EJ, Woster AP, DeWitt P, Goldstein RS, Levy K. 2016. A systematic review and meta-analysis of ambient temperature and diarrhoeal diseases. International Journal of Epidemiology 45:117-130.

Centers for Disease Control and Prevention. 2014. National *Salmonella* Surveillance Annual Report, 2012. Atlanta, Georgia: US Department of Health and Human Services, CDC.

Centers for Disease Control and Prevention. 2015. Serotypes and the Importance of Serotyping *Salmonella*. Available: https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html [accessed 21 February 2018].

Centers for Disease Control and Prevention. 2016a. PulseNet: 20 years of making food safer to eat. Available: https://www.cdc.gov/pulsenet/anniversary/20-years.html [accessed March 29 2018]

Centers for Disease Control and Prevention. 2016b. National *Salmonella* Surveillance Annual Report, 2013. Atlanta, Georgia:US Department of Health and Human Services, CDC.

Centers for Disease Control and Prevention. 2017a. National Enteric Disease Surveillance: *Salmonella* Annual Report, 2015. Atlanta, GA.

Centers for Disease Control and Prevention. 2017b. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). Atlanta, Georgia:U.S. Department of Health and Human Services, CDC.

Cerna-Cortes JF, Gómez-Aldapa CA, Rangel-Vargas E, Ramírez-Cruz E, Castro-Rosas J. 2013. Presence of indicator bacteria, *Salmonella* and diarrheagenic *Escherichia coli*

pathotypes on mung bean sprouts from public markets in Pachuca, Mexico. Food Control 31:280-283.

Cevallos-Cevallos JM, Danyluk MD, Gu GY, Vallad GE, van Bruggen AHC. 2012. Dispersal of *Salmonella* Typhimurium by Rain Splash onto Tomato Plants. J Food Prot 75:472-479.

Chiu CH, Ou JT. 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. Journal of Clinical Microbiology 34:2619-2622.

Cohen J. 1960. A Coefficient of Agreement for Nominal Scales. Educational and Psychological Measurement 20:37-46.

Crim SM, Griffin PM, Tauxe R, Marder EP, Gilliss D, Cronquist AB, et al. 2015. Preliminary Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food - Foodborne Diseases Active Surveillance Network, 10 US Sites, 2006-2014. MMWR-Morb Mortal Wkly Rep 64:495-499.

Curriero FC, Patz JA, Rose JB, Lele S. 2001. The Association Between Extreme Precipitation and Waterborne Disease Outbreaks in the United States, 1948–1994. American Journal of Public Health 91:1194-1199.

Dai A. 2012. Increasing drought under global warming in observations and models. Nature Climate Change 3:52.

Danyluk M, Nozawa-Inoue M, Hristova K, Scow K, Lampinen B, Harris L. 2008. Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. Journal of applied microbiology 104:1391-1399.

Danyluk MD, Schaffner DW. 2011. Quantitative Assessment of the Microbial Risk of Leafy Greens from Farm to Consumption: Preliminary Framework, Data, and Risk Estimates. J Food Prot 74:700-708.

Davis K, Anderson MA, Yates MV. 2005. Distribution of indicator bacteria in Canyon Lake, California. Water Res 39:1277-1288.

Decol LT, Casarin LS, Hessel CT, Batista ACF, Allende A and Tondo EC. 2017. Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. Food Microbiology 65:105-113.

Deng W, Liou SR, Plunkett G, Mayhew GF, Rose DJ, Burland V, et al. 2003. Comparative genomics of *Salmonella enterica* serovar typhi strains Ty2 and CT18. Journal of Bacteriology 185:2330-2337.

Deng X, Shariat N, Driebe EM, Roe CC, Tolar B, Trees E, et al. 2015. Comparative Analysis of Subtyping Methods against a Whole-Genome-Sequencing Standard for *Salmonella enterica* Serotype Enteritidis. Journal of Clinical Microbiology 53:212-218.

Detty JM, McGuire KJ. 2010. Threshold changes in storm runoff generation at a till - mantled headwater catchment. Water Resources Research 46.

Efstratiou M, Mavridou A, Richardson S, Papadakis J. 1998. Correlation of bacterial indicator organisms with *Salmonella* spp., *Staphylococcus aureus* and *Candida albicans* in sea water. Letters in applied microbiology 26:342-346.

Erickson MC, Habteselassie MY, Liao J, Webb CC, Mantripragada V, Davey LE, et al. 2014. Examination of factors for use as potential predictors of human enteric pathogen survival in soil. Journal of Applied Microbiology 116:335-349.

Erickson MC. 2016. Overview: Foodborne Pathogens in Wildlife Populations. Cham:Springer Int Publishing Ag.

Erickson MC, Liao J-Y, Webb CC, Habteselassie MY, Cannon JL. 2018a. Efficacy of chlorine as a disinfecting agent on produce-harvesting gloves contaminated with *Escherichia coli* O157:H7 or *Salmonella*. Food Control 86:257-265.

Erickson MC, Liao J-Y, Webb CC, Habteselassie MY, Cannon JL. 2018b. Inactivation of *Escherichia coli* O157:H7 and *Salmonella* deposited on gloves in a liquid state and subjected to drying conditions. International Journal of Food Microbiology 266:200-206.

Falardeau J, Johnson RP, Pagotto F, Wang S. 2017. Occurrence, characterization, and potential predictors of verotoxigenic *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in surface water used for produce irrigation in the Lower Mainland of British Columbia, Canada. PLoS One 12:e0185437.

Ferguson AS, Layton AC, Mailloux BJ, Culligan PJ, Williams DE, Smartt AE, et al. 2012. Comparison of fecal indicators with pathogenic bacteria and rotavirus in groundwater. The Science of the total environment 431:314-322.

Fischer EM, Knutti R. 2016. Observed heavy precipitation increase confirms theory and early models. Nature Climate Change 6:986.

Fohrer N, Berkenhagen J, Hecker JM, Rudolph A. 1999. Changing soil and surface conditions during rainfall: Single rainstorm/subsequent rainstorms. CATENA 37:355-375.

Food and Drug Administration. 2015. Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption.

Foster JW, Spector MP. 1995. How *Salmonella* Survive Against the Odds. Annual Review of Microbiology 49:145-174.

Franz E, van Bruggen AHC. 2008. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the Primary Vegetable Production Chain. Crit Rev Microbiol 34:143-161.

Franz E, Visser AA, Van Diepeningen AD, Klerks MM, Termorshuizen AJ, van Bruggen AHC. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiology 24:106-112.

Frauenfeld B, Truman C. 2004. Variable rainfall intensity effects on runoff and interrill erosion from two coastal plain ultisols in Georgia. Soil Sci 169:143-154.

Gaul SB, Wedel S, Erdman MM, Harris DL, Harris IT, Ferris KE, et al. 2007. Use of Pulsed-Field Gel Electrophoresis of Conserved *XbaI* Fragments for Identification of Swine *Salmonella* Serotypes. Journal of Clinical Microbiology 45:472-476.

Gelting RJ and Baloch M. 2013. A Systems Analysis of Irrigation Water Quality in Environmental Assessments Related to Foodborne Outbreaks. Aquatic Procedia 1:130-137.

Gelting RJ, Baloch MA, Zarate-Bermudez MA, Selman C. 2011. Irrigation water issues potentially related to the 2006 multistate *E. coli* O157:H7 outbreak associated with spinach. Agricultural Water Management 98:1395-1402.

Georgia Department of Public Health. 2015. Data Request, Public Health Information Portal. Atlanta, Georgia [accessed December 4 2017].

Georgia Department of Public Health. 2018. Population Web Query, Online Analytical Statistical Information System [accessed December 18 2017].

Gerstel U, Römling U. 2001. Oxygen tension and nutrient starvation are major signals that regulate agfD promoter activity and expression of the multicellular morphotype in *Salmonella* typhimurium. Environmental Microbiology 3:638-648.

Gil MI, Selma MV, Suslow T, Jacxsens L, Uyttendaele M, Allende A. 2015. Pre- and Postharvest Preventive Measures and Intervention Strategies to Control Microbial Food Safety Hazards of Fresh Leafy Vegetables. Critical Reviews in Food Science and Nutrition 55:453-468.

Gomba A, Chidamba L, Korsten L. 2016. Prevalence and serovar diversity of *Salmonella* spp. in primary horticultural fruit production environments. Food Control 69:13-19.

Gorski L, Parker CT, Liang A, Cooley MB, Jay-Russell MT, Gordus AG, et al. 2011. Prevalence, Distribution, and Diversity of *Salmonella enterica* in a Major Produce Region of California. Applied and Environmental Microbiology 77:2734-2748.

Greene SK, Daly ER, Talbot EA, Demma LJ, Holzbauer S, Patel NJ, et al. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. Epidemiol Infect 136:157-165.

Grjibovski AM, Bushueva V, Boltenkov VP, Buzinov RV, Degteva GN, Yurasova ED, et al. 2012. Climate variations and salmonellosis in northwest Russia: a time-series analysis. Epidemiol Infect 141:269-276.

Grjibovski AM, Kosbayeva A, Menne B. 2013. The effect of ambient air temperature and precipitation on monthly counts of salmonellosis in four regions of Kazakhstan, Central Asia, in 2000–2010. Epidemiol Infect 142:608-615.

Grocery Manufacturers Association, Food Marketing Institute, GS1 US, Deloitte. 2010. Recall Execution Effectiveness: Collaborative Approaches to Improving Consumer Safety and Confidence.

Grocery Manufacturers Association. 2011. Capturing Recall Costs: Measuring and Recovering the Losses.

Gu GY, Luo ZY, Cevallos-Cevallos JM, Adams P, Vellidis G, Wright A, et al. 2013. Factors affecting the occurrence of *Escherichia coli* O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed. Can J Microbiol 59:175-182.

Haley BJ, Cole DJ, Lipp EK. 2009. Distribution, Diversity, and Seasonality of Waterborne Salmonellae in a Rural Watershed. Applied and Environmental Microbiology 75:1248-1255.

Hamidin N, Yu QJ, Connell DW. 2008. Human health risk assessment of chlorinated disinfection by-products in drinking water using a probabilistic approach. Water Res 42:3263-3274.

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 6:635-648.

Harris CS, Tertuliano M, Rajeev S, Vellidis G, Levy K. 2018. Impact of storm runoff on *Salmonella* and *Escherichia coli* prevalence in irrigation ponds of fresh produce farms in southern Georgia. Journal of Applied Microbiology 124:910-921.

Harwood VJ, Levine AD, Scott TM, Chivukula V, Lukasik J, Farrah SR, et al. 2005. Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection. Applied and Environmental Microbiology 71:3163-3170.

Hashizume M, Armstrong B, Hajat S, Wagatsuma Y, Faruque ASG, Hayashi T, et al. 2007. Association between climate variability and hospital visits for non-cholera diarrhoea in Bangladesh: effects and vulnerable groups. International Journal of Epidemiology 36:1030-1037.

Henao OL, Jones TF, Vugia DJ, Griffin PM, for the Foodborne Diseases Active Surveillance Network W. 2015. Foodborne Diseases Active Surveillance Network—2 Decades of Achievements, 1996–2015. Emerg Infect Dis 21:1529-1536.

Hirneisen KA, Sharma M, Kniel KE. 2012. Human Enteric Pathogen Internalization by Root Uptake into Food Crops. Foodborne Pathog Dis 9:396-405.

Hoelzer K, Moreno Switt AI, Wiedmann M. 2011. Animal contact as a source of human non-typhoidal salmonellosis. Veterinary Research 42:34-34.

Hoffman S, Maculloch B, Batz M. 2015. Economic Burden of Major Foodborne Illnesses Acquired in the United States. EIB-140.U.S. Department of Agriculture, Economic Research Service.

Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. 2006. *Salmonella* Survival in Manure-Treated Soils during Simulated Seasonal Temperature Exposure. Journal of Environmental Quality 35:1170-1180.

Huang AD, Luo C, Pena-Gonzalez A, Weigand MR, Tarr CL, Konstantinidis KT. 2017. Metagenomics of Two Severe Foodborne Outbreaks Provides Diagnostic Signatures and Signs of Coinfection Not Attainable by Traditional Methods. Applied and Environmental Microbiology 83.

Huang JY, Henao OL, Griffin PM, Vugia DJ, Cronquist AB, Hurd S, et al. 2016. Infection with Pathogens Transmitted Commonly Through Food and the Effect of Increasing Use of Culture-Independent Diagnostic Tests on Surveillance - Foodborne Diseases Active Surveillance Network, 10 US Sites, 2012-2015. MMWR-Morb Mortal Wkly Rep 65:368-371.

Huang JY, Patrick ME, Manners J, Sapkota AR, Scherzinger KJ, Tobin-D'Angelo M, et al. 2017. Association between wetland presence and incidence of *Salmonella enterica*

serotype Javiana infections in selected US sites, 2005–2011. Epidemiol Infect 145:2991-2997.

Hudson CR, Quist C, Lee MD, Keyes K, Dodson SV, Morales C, et al. 2000. Genetic relatedness of *Salmonella* isolates from nondomestic birds in southeastern United States. Journal of Clinical Microbiology 38:1860-1865.

Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang XP. 2004. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. Applied and Environmental Microbiology 70:2497-2502.

Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2005. Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. Food Microbiology 22:63-70.

Jackson BR, Griffin PM, Cole D, Walsh KA, Chai SJ. 2013. Outbreak-associated *Salmonella enterica* Serotypes and Food Commodities, United States, 1998–2008. Emerg Infect Dis 19:1239.

Jacobsen CS, Bech TB. 2012. Soil survival of *Salmonella* and transfer to freshwater and fresh produce. Food Research International 45:557-566.

Jagai JS, Li QL, Wang S, Messier KP, Wade TJ, Hilborn ED. 2015. Extreme Precipitation and Emergency Room Visits for Gastrointestinal Illness in Areas with and without Combined Sewer Systems: An Analysis of Massachusetts Data, 2003-2007. Environ Health Perspect 123:873-879.

Jenkins MB, Endale DM, Fisher DS, Paige Adams M, Lowrance R, Larry Newton G, et al. 2012. Survival dynamics of fecal bacteria in ponds in agricultural watersheds of the Piedmont and Coastal Plain of Georgia. Water Res 46:176-186.

Jenkinson P, Parry DW. 1994. Splash dispersal of conidia of *Fusarium culmorum* and *Fusarium avenaceum*. Mycological Research 98:506-510.

Jeong CH, Wagner ED, Siebert VR, Anduri S, Richardson SD, Daiber EJ, et al. 2012. Occurrence and Toxicity of Disinfection Byproducts in European Drinking Waters in Relation with the HIWATE Epidemiology Study. Environmental Science & Technology 46:12120-12128.

Jia S, Shi P, Hu Q, Li B, Zhang T, Zhang X-X. 2015. Bacterial Community Shift Drives Antibiotic Resistance Promotion during Drinking Water Chlorination. Environmental Science & Technology 49:12271-12279.

Jiang C, Shaw KS, Upperman CR, Blythe D, Mitchell C, Murtugudde R, et al. 2015. Climate change, extreme events and increased risk of salmonellosis in Maryland, USA: Evidence for coastal vulnerability. Environment International 83:58-62.

Jokinen CC, Schreier H, Mauro W, Taboada E, Isaac-Renton JL, Topp E, Edge T, Thomas JE and Gannon VPJ. 2010. The occurrence and sources of *Campylobacter* spp., *Salmonella enterica* and *Escherichia coli* O157:H7 in the Salmon River, British Columbia, Canada. J Water Health 8:374-386.

Jokinen C, Edge TA, Ho S, Koning W, Laing C, Mauro W, et al. 2011. Molecular subtypes of *Campylobacter* spp., *Salmonella enterica*, and *Escherichia coli* O157:H7 isolated from faecal and surface water samples in the Oldman River watershed, Alberta, Canada. Water Res 45:1247-1257.

Jung Y, Jang H, Matthews KR. 2014. Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. Microbial Biotechnology 7:517-527.

Kahler MA, Johnson BT, Hahn D, Narayanan J, Derado G, Hill RV. 2015. Evaluation of an Ultrafiltration-Based Procedure for Simultaneous Recovery of Diverse Microbes in Source Waters. Water 7.

Keraita B, Konradsen F, Drechsel P, Abaidoo RC. 2007. Reducing microbial contamination on wastewater-irrigated lettuce by cessation of irrigation before harvesting. Tropical Medicine & International Health 12:8-14.

Kibet LC, Saporito LS, Allen AL, May EB, Kleinman PJA, Hashem FM, et al. 2014. A Protocol for Conducting Rainfall Simulation to Study Soil Runoff. Journal of Visualized Experiments : JoVE:51664.

Kisluk G, Yaron S. 2012. Presence and Persistence of *Salmonella enterica* Serotype Typhimurium in the Phyllosphere and Rhizosphere of Spray-Irrigated Parsley. Applied and Environmental Microbiology 78:4030-4036.

Kunkel KE, Stevens LE, Stevens SE, Sun L, Janssen E, Wuebbles D, et al. 2013. Regional Climate Trends and Scenarios for the US National Climate Assessment: Part 2. Climate of the Southeast US.National Oceanic and Atmospheric Administration.

Labbe RG. 2013. Guide to Foodborne Pathogens. Part 2nd (Labbe RG, Garcia S, eds). Chichester, West Sussex, UK:Wiley Blackwell.

Landis JR, Koch GG. 1977. The Measurement of Observer Agreement for Categorical Data. Biometrics 33:159-174.

Lang NL, Smith SR. 2007. Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions. Journal of Applied Microbiology 103:2122-2131.

Langley G, Besser J, Iwamoto M, Lessa FC, Cronquist A, Skoff TH, et al. 2015. Effect of Culture-Independent Diagnostic Tests on Future Emerging Infections Program Surveillance. Emerg Infect Dis 21:1582-1588.

Lee H, Lau S-L, Kayhanian M, Stenstrom MK. 2004. Seasonal first flush phenomenon of urban stormwater discharges. Water Res 38:4153-4163.

Levantesi C, Bonadonna L, Briancesco R, Grohmann E, Toze S, Tandoi V. 2012. *Salmonella* in surface and drinking water: Occurrence and water-mediated transmission. Food Research International 45:587-602.

Levy K, Hubbard AE, Nelson KL, Eisenberg JNS. 2009. Drivers of Water Quality Variability in Northern Coastal Ecuador. Environmental science & technology 43:1788-1797.

Levy K, Woster AP, Goldstein RS, Carlton EJ. 2016. Untangling the Impacts of Climate Change on Waterborne Diseases: a Systematic Review of Relationships between Diarrheal Diseases and Temperature, Rainfall, Flooding, and Drought. Environmental Science & Technology 50:4905-4922.

Li BG, Vellidis G, Liu HL, Jay-Russell M, Zhao SH, Hu ZL, et al. 2014. Diversity and Antimicrobial Resistance of *Salmonella enterica* Isolates from Surface Water in Southeastern United States. Applied and Environmental Microbiology 80:6355-6365.

Li BG, Jackson SA, Gangiredla J, Wang WM, Liu HL, Tall BD, Beaubrun JJG, Jay-Russell M, Vellidis G and Elkins CA. 2015. Genomic Evidence Reveals Numerous *Salmonella enterica* Serovar Newport Reintroduction Events in Suwannee Watershed Irrigation Ponds. Applied and Environmental Microbiology 81:8243-8253.

Li X-F, Mitch WA. 2018. Drinking Water Disinfection Byproducts (DBPs) and Human Health Effects: Multidisciplinary Challenges and Opportunities. Environmental Science & Technology 52:1681-1689.

Lind AJ. 2008. Amphibians and Reptiles and Climate Change.U.S. Department of Agriculture, Forest Service, Climate Change Resource Center.

Lipp EK, Kurz R, Vincent R, Rodriguez-Palacios C, Farrah SR, Rose JB. 2001. The Effects of Seasonal Variability and Weather on Microbial Fecal Pollution and Enteric Pathogens in a Subtropical Estuary. Estuaries 24:266-276.

Liu C, Hofstra N, Franz E. 2013. Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp. International Journal of Food Microbiology 163:119-128.

Liu H, Lei TW, Zhao J, Yuan CP, Fan YT, Qu LQ. 2011. Effects of rainfall intensity and antecedent soil water content on soil infiltrability under rainfall conditions using the run off-on-out method. Journal of Hydrology 396:24-32.

Luo Z, Gu G, Giurcanu MC, Adams P, Vellidis G, van Bruggen AHC, et al. 2014. Development of a novel cross-streaking method for isolation, confirmation, and enumeration of *Salmonella* from irrigation ponds. Journal of Microbiological Methods 101:86-92.

Luo Z, Gu G, Ginn A, Giurcanu MC, Adams P, Vellidis G, et al. 2015. Distribution and Characterization of *Salmonella enterica* Isolates from Irrigation Ponds in the Southeastern United States. Applied and Environmental Microbiology 81:4376-4387.

Ma B, Yu X, Ma F, Li Z, Wu F. 2014. Effects of Crop Canopies on Rain Splash Detachment. PLoS One 9:e99717.

Madden L. 1997. Effects of rain on splash dispersal of fungal pathogens. Canadian Journal of Plant Pathology 19:225-230.

Maffei DF, Sant'Ana AS, Franco BDGM, Schaffner DW. 2017. Quantitative assessment of the impact of cross-contamination during the washing step of ready-to-eat leafy greens on the risk of illness caused by *Salmonella*. Food Research International 92:106-112.

Mandrell RE. 2009. Enteric Human Pathogens Associated with Fresh Produce: Sources, Transport and Ecology. In: Microbial Safety of Fresh Produce, (Fan X, Niemira BA, Doona CJ, Feeherry FE, Gravani RB, eds):IFT Press, 5-42.

Marder EP, Cieslak PR, Cronquist AB, Dunn J, Lathrop S, Rabatsky-Ehr T, et al. 2017. Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food and the Effect of Increasing Use of Culture-Independent Diagnostic Tests on Surveillance — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2013–2016. MMWR Morb Mortal Wkly Rep 66:397–403.

Maurer JJ, Martin G, Hernandez S, Cheng Y, Gerner-Smidt P, Hise KB, et al. 2015. Diversity and Persistence of *Salmonella enterica* Strains in Rural Landscapes in the Southeastern United States. PLoS One 10:e0128937.

Mawdsley JL, Bardgett RD, Merry RJ, Pain BF, Theodorou MK. 1995. Pathogens in livestock waste, their potential for movement through soil and environmental pollution. Applied Soil Ecology 2:1-15.

McEgan R, Mootian G, Goodridge LD, Schaffner DW and Danyluk MD. 2013. Predicting *Salmonella* Populations from Biological, Chemical, and Physical Indicators in Florida Surface Waters. Applied and Environmental Microbiology 79:4094-4105.

Micallef SA, Rosenberg Goldstein RE, George A, Kleinfelter L, Boyer MS, McLaughlin CR, et al. 2012. Occurrence and antibiotic resistance of multiple *Salmonella* serotypes recovered from water, sediment and soil on mid-Atlantic tomato farms. Environmental Research 114:31-39.

Monaghan JM, Hutchison ML. 2012. Distribution and decline of human pathogenic bacteria in soil after application in irrigation water and the potential for soil-splash-mediated dispersal onto fresh produce. Journal of Applied Microbiology 112:1007-1019.

Mootian G, Wu W-H, Matthews KR. 2009. Transfer of *Escherichia coli* O157:H7 from Soil, Water, and Manure Contaminated with Low Numbers of the Pathogen to Lettuce Plants. J Food Prot 72:2308-2312.

Mualem Y, Assouline S, Rohdenburg H. 1990. Rainfall Induced Soil Seal. A Critical-Review of Observations and Models. Catena 17:185-203.

Mughini-Gras L, Enserink R, Friesema I, Heck M, van Duynhoven Y, van Pelt W. 2014. Risk Factors for Human Salmonellosis Originating from Pigs, Cattle, Broiler Chickens and Egg Laying Hens: A Combined Case-Control and Source Attribution Analysis. PLoS One 9:e87933.

Mugnai R, Sattamini A, Albuquerque dos Santos JA, Regua-Mangia AH. 2015. A Survey of *Escherichia coli* and *Salmonella* in the Hyporheic Zone of a Subtropical Stream: Their Bacteriological, Physicochemical and Environmental Relationships. PLoS One 10:e0129382.

Mull B, Hill VR. 2009. Recovery and Detection of *Escherichia coli* O157:H7 in Surface Water, Using Ultrafiltration and Real-Time PCR. Applied and Environmental Microbiology 75:3593-3597.

Mull B, Hill VR. 2012. Recovery of diverse microbes in high turbidity surface water samples using dead-end ultrafiltration. Journal of Microbiological Methods 91:429-433.

Murray K, Wu F, Shi J, Jun Xue S, Warriner K. 2017. Challenges in the microbiological food safety of fresh produce: Limitations of post-harvest washing and the need for alternative interventions. Food Quality and Safety 1:289-301.

Nautiyal CS, Rehman A, Chauhan PS. 2010. Environmental *Escherichia coli* occur as natural plant growth-promoting soil bacterium. Archives of Microbiology 192:185-193.

Nichols G, Lane C, Asgari N, Verlander NQ, Charlett A. 2009. Rainfall and outbreaks of drinking water related disease and in England and Wales. J Water Health 7:1-8.

NOAA National Climatic Data Center. 2018. Climate Data Online [accessed December 4 2017].

Ntahimpera N, Ellis M, Wilson L, Madden L. 1998. Effects of a cover crop on splash dispersal of *Colletotrichum acutatum* conidia. Phytopathology 88:536-543.

Nyberg KA, Ottoson JR, Vinnerås B, Albihn A. 2014. Fate and survival of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in repacked soil lysimeters after application of cattle slurry and human urine. Journal of the Science of Food and Agriculture 94:2541-2546.

Office of Disease Prevention and Health Promotion. 2010. Food Safety. Available: https://www.healthypeople.gov/2020/topics-objectives/topic/food-safety [accessed March 14 2018].

Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiology 32:1-19.

Oliveira M, Viñas I, Usall J, Anguera M, Abadias M. 2012. Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated

compost and irrigation water. International Journal of Food Microbiology 156:133-140.

Pachepsky YA, Shelton DR, McLain JET, Patel J, Mandrell RE. 2011. Chapter Two - Irrigation Waters as a Source of Pathogenic Microorganisms in Produce: A Review. In: Advances in Agronomy, Vol. Volume 113, (Donald LS, ed):Academic Press, 75-141.

Pachepsky YA, Morrow J, Guber A, Shelton D, Rowland R, Davies G. 2012. Effect of biofilm in irrigation pipes on microbial quality of irrigation water. Letters in Applied Microbiology 54:217-224.

Pachepsky YA, Blaustein RA, Whelan G, Shelton DR. 2014. Comparing temperature effects on *Escherichia coli*, *Salmonella*, and *Enterococcus* survival in surface waters. Letters in Applied Microbiology 59:278-283.

Pachepsky YA, Shelton D, Dorner S and Whelan G. 2016. Can *E. coli* or thermotolerant coliform concentrations predict pathogen presence or prevalence in irrigation waters? Crit Rev Microbiol 42:384-393.

Pachepsky YA, Kierzewski R, Stocker M, Sellner K, Mulbry W, Lee H, et al. 2018. Temporal Stability of Escherichia coli Concentrations in Waters of Two Irrigation Ponds in Maryland. Applied and Environmental Microbiology 84.

Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, et al. 2013. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998-2008. Emerg Infect Dis 19:407-415.

Park S, Szonyi B, Gautam R, Nightingale K, Anciso J, Ivanek R. 2012. Risk Factors for Microbial Contamination in Fruits and Vegetables at the Preharvest Level: A Systematic Review. J Food Prot 75:2055-2081.

Park S-H, Kang D-H. 2015. Combination treatment of chlorine dioxide gas and aerosolized sanitizer for inactivating foodborne pathogens on spinach leaves and tomatoes. International Journal of Food Microbiology 207:103-108.

Parlak M, Parlak AÖ. 2010. Measurement of splash erosion in different cover crops. Turkish journal of field crops 15:169-173.

Partyka ML, Bond RF, Chase JA, Atwill ER. 2017. Monitoring bacterial indicators of water quality in a tidally influenced delta: A Sisyphean pursuit. Science of The Total Environment 578:346-356.

Partyka ML, Bond RF, Chase JA, Atwill ER. 2018. Spatial and temporal variability of bacterial indicators and pathogens in six California reservoirs during extreme drought. Water Res 129:436-446.

Payment P, Locas A. 2011. Pathogens in Water: Value and Limits of Correlation with Microbial Indicators. Ground Water 49:4-11.

Penet L, Guyader S, Pétro D, Salles M, Bussière F. 2014. Direct Splash Dispersal Prevails over Indirect and Subsequent Spread during Rains in *Colletotrichum gloeosporioides* Infecting Yams. PLoS One 9:e115757.

Penna D, Tromp-van Meerveld H, Gobbi A, Borga M, Dalla Fontana G. 2011. The influence of soil moisture on threshold runoff generation processes in an alpine headwater catchment. Hydrology and Earth System Sciences 15:689.

Pennington W. 2014. Establishing a Method for Microbiological Evaluation of Fresh Produce at Risk for *Salmonella* Contamination. Emory University.

Pires AFA, Funk JA, Lim A, Bolin SR. 2013. Enumeration of *Salmonella* in Feces of Naturally Infected Pigs. Foodborne Pathog Dis 10:933-937.

Pires SM, Vieira AR, Perez E, Wong DLF, Hald T. 2012. Attributing human foodborne illness to food sources and water in Latin America and the Caribbean using data from outbreak investigations. International Journal of Food Microbiology 152:129-138.

Prein AF, Rasmussen RM, Ikeda K, Liu C, Clark MP, Holland GJ. 2016. The future intensification of hourly precipitation extremes. Nature Climate Change 7:48.

Quested TE, Cook PE, Gorris LGM, Cole MB. 2010. Trends in technology, trade and consumption likely to impact on microbial food safety. International Journal of Food Microbiology 139:S29-S42.

R Core Team. 2015. R: A language and environment for statistical computing. Vienna, Austria:R Foundation for Statistical Computing.

Rajwar A, Srivastava P, Sahgal M. 2016. Microbiology of Fresh Produce: Route of Contamination, Detection Methods, and Remedy. Critical Reviews in Food Science and Nutrition 56:2383-2390.

Reddy SP, Wang H, Adams JK, Feng PCH. 2016. Prevalence and Characteristics of *Salmonella* Serotypes Isolated from Fresh Produce Marketed in the United States. J Food Prot 79:6-16.

Refsum T, Heir E, Kapperud G, Vardund T, Holstad G. 2002. Molecular Epidemiology of Salmonella enterica Serovar Typhimurium Isolates Determined by Pulsed-Field Gel Electrophoresis: Comparison of Isolates from Avian Wildlife, Domestic Animals, and the Environment in Norway. Applied and Environmental Microbiology 68:5600-5606.

Reller ME, Tauxe RV, Kalish LA, MØLbak K. 2007. Excess salmonellosis in women in the United States: 1968–2000. Epidemiol Infect 136:1109-1117.

Rodgers P, Soulsby C, Hunter C, Petry J. 2003. Spatial and temporal bacterial quality of a lowland agricultural stream in northeast Scotland. Science of The Total Environment 314-316:289-302.

Rose JB, Daeschner S, Easterling DR, Curriero FC, et al. 2000. Climate and waterborne disease outbreaks. American Water Works Association Journal 92:77.

Rozen Y, Belkin S. 2001. Survival of enteric bacteria in seawater. FEMS microbiology reviews 25:513-529.

Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. 2011a. Foodborne Illness Acquired in the United States—Unspecified Agents. Emerging Infectious Disease journal 17:16.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, et al. 2011b. Foodborne Illness Acquired in the United States—Major Pathogens. Emerg Infect Dis 17:7-15.

Semenov AV, van Bruggen AHC, van Overbeek L, Termorshuizen AJ, Semenov AM. 2007. Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure. FEMS Microbiol Ecol 60:419-428.

Semenov AV, van Overbeek L, van Bruggen AHC. 2009. Percolation and Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium in Soil Amended with Contaminated Dairy Manure or Slurry. Applied and Environmental Microbiology 75:3206-3215.

Semenza JC, Herbst S, Rechenburg A, Suk JE, Höser C, Schreiber C, et al. 2012. Climate Change Impact Assessment of Food- and Waterborne Diseases. Critical Reviews in Environmental Science and Technology 42:857-890.

Shah DH, Paul NC, Sischo WC, Crespo R, Guard J. 2017. Population dynamics and antimicrobial resistance of the most prevalent poultry-associated *Salmonella* serotypes. Poultry Science 96:687-702.

Sharma M, Ingram DT, Patel JR, Millner PD, Wang X, Hull AE, et al. 2009. A Novel Approach To Investigate the Uptake and Internalization of *Escherichia coli* O157:H7 in Spinach Cultivated in Soil and Hydroponic Medium. J Food Prot 72:1513-1520.

Singh RB, Hales S, de Wet N, Raj R, Hearnden M, Weinstein P. 2001. The influence of climate variation and change on diarrheal disease in the Pacific Islands. Environ Health Perspect 109:155-159.

Sivapalasingam S, Friedman CR, Cohen L, Tauxe RV. 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J Food Prot 67:2342-2353.

Sowah R, Zhang H, Radcliffe D, Bauske E, Habteselassie MY. 2014. Evaluating the influence of septic systems and watershed characteristics on stream faecal pollution in suburban watersheds in Georgia, USA. Journal of Applied Microbiology 117:1500-1512.

Spector MP, Kenyon WJ. 2012. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. Food Research International 45:455-481.

Srikantiah P, Lay JC, Hand S, Crump JA, Campbell J, Van Duyne MS, et al. 2004. *Salmonella enterica* serotype Javiana infections associated with amphibian contact, Mississippi, 2001. Epidemiol Infect 132:273-281.

Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688-2690.

Stearn DW. 2018. Safety of the U.S. Food Supply: Continuing Concerns Over The Food and Drug Administration's Food Recall Process. In: Subcommittee on Oversight and Investigations, Committee on Energy and Commerce, US House of Representatives:U.S. Food and Drug Administration.

Steele M, Odumeru J. 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J Food Prot 67:2839-2849.

Stephen DM, Barnett AG. 2016. Effect of temperature and precipitation on salmonellosis cases in South-East Queensland, Australia: an observational study. BMJ Open 6.

Stine SW, Song IH, Choi CY, Gerba CP. 2005. Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce. J Food Prot 68:913-918.

Strawn LK, Fortes ED, Bihn EA, Nightingale KK, Gröhn YT, Worobo RW, et al. 2013a. Landscape and Meteorological Factors Affecting Prevalence of Three Food-Borne Pathogens in Fruit and Vegetable Farms. Applied and Environmental Microbiology 79:588-600.

Strawn LK, Gröhn YT, Warchocki S, Worobo RW, Bihn EA, Wiedmann M. 2013b. Risk Factors Associated with *Salmonella* and *Listeria monocytogenes* Contamination of Produce Fields. Applied and Environmental Microbiology 79:7618-7627.

Strawn LK, Danyluk MD, Worobo RW, Wiedmann M. 2014. Distribution of *Salmonella* Subtypes Differs Between Two US Produce Growing Regions. Applied and Environmental Microbiology.

Suslow T. 2005. Chlorination in the production and postharvest handling of fresh fruits and vegetables.

Suslow TV. 2003. Key Points of Control and Management for Microbial Food Safety: Information for Growers, Packers, and Handlers of Fresh-Consumed Horticultural Products.

Tallon LK, Si BC, Korber D, Guo X. 2007. Soil wetting state and preferential transport of *Escherichia coli* in clay soils. Can J Soil Sci 87:61-72.

Tauxe RV, Doyle MP, Kuchenmüller T, Schlundt J, Stein CE. 2010. Evolving public health approaches to the global challenge of foodborne infections. International Journal of Food Microbiology 139:S16-S28.

Thomas JL, Slawson RM, Taylor WD. 2013. *Salmonella* serotype diversity and seasonality in urban and rural streams. Journal of Applied Microbiology 114:907-922.

Thomas MK, Charron DF, Waltner-Toews D, Schuster C, Maarouf AR, Holt JD. 2006. A role of high impact weather events in waterborne disease outbreaks in Canada, 1975 – 2001. International Journal of Environmental Health Research 16:167-180.

Tomás-Callejas A, López-Velasco G, Camacho AB, Artés F, Artés-Hernández F, Suslow TV. 2011. Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. International Journal of Food Microbiology 151:216-222.

Trenberth KE. 2011. Changes in precipitation with climate change. Climate Research 47:123-138.

Trinetta V, Vaidya N, Linton R, Morgan M. 2011. Evaluation of Chlorine Dioxide Gas Residues on Selected Food Produce. J Food Sci 76:T11-T15.

Truman CC, Bradford JM. 1990. Effect of antecedent soil moisture on splash detachment under simulated rainfall. Soil Sci 150:787-798.

Truman CC, Strickland TC, Potter TL, Franklin DH, Bosch DD, Bednarz CW. 2007. Variable Rainfall Intensity and Tillage Effects on Runoff, Sediment, and Carbon Losses from a Loamy Sand under Simulated Rainfall. Journal of Environmental Quality 36:1495-1502.

Truman CC, Potter TL, Nuti RC, Franklin DH, Bosch DD. 2011. Antecedent water content effects on runoff and sediment yields from two Coastal Plain Ultisols. Agricultural Water Management 98:1189-1196.

Underthun K, De J, Gutierrez A, Silverberg R, Schneider KR. 2018. Survival of *Salmonella* and *Escherichia coli* in Two Different Soil Types at Various Moisture Levels and Temperatures. J Food Prot 81:150-157.

USDA National Agricultural Statistics Service. 2017. Vegetables 2016 Summary (February 2017).

Uyttendaele M, Jaykus L-A, Amoah P, Chiodini A, Cunliffe D, Jacxsens L, et al. 2015. Microbial Hazards in Irrigation Water: Standards, Norms, and Testing to Manage Use of Water in Fresh Produce Primary Production. Comprehensive Reviews in Food Science and Food Safety 14:336-356.

Van der Linden I, Cottyn B, Uyttendaele M, Vlaemynck G, Heyndrickx M, Maes M. 2013. Survival of Enteric Pathogens During Butterhead Lettuce Growth: Crop Stage, Leaf Age, and Irrigation. Foodborne Pathog Dis 10:485-491.

Van Elsas JD, Trevors JT, Van Overbeek LS. 1991. Influence of Soil Properties on the Vertical Movement of Genetically-Marked *Pseudomonas*-Fluorescens Through Large Soil Microcosms. Biol Fertil Soils 10:249-255.

Vereen Jr E, Lowrance RR, Jenkins MB, Adams P, Rajeev S, Lipp EK. 2013. Landscape and seasonal factors influence *Salmonella* and *Campylobacter* prevalence in a rural mixed use watershed. Water Res 47:6075-6085.

Verhougstraete MP, Martin SL, Kendall AD, Hyndman DW, Rose JB. 2015. Linking fecal bacteria in rivers to landscape, geochemical, and hydrologic factors and sources at the basin scale. Proceedings of the National Academy of Sciences.

Wadamori Y, Gooneratne R and Hussain MA. 2017. Outbreaks and factors influencing microbiological contamination of fresh produce. Journal of the Science of Food and Agriculture 97:1396-1403.

Waldner LL, MacKenzie KD, Köster W, White AP. 2012. From exit to entry: long-term survival and transmission of *Salmonella*. Pathogens 1:128-155.

Walls SC, Barichivich WJ, Brown ME, Scott DE, Hossack BR. 2013. Influence of Drought on Salamander Occupancy of Isolated Wetlands on the Southeastern Coastal Plain of the United States. Wetlands 33:345-354.

Walters SP, González-Escalona N, Son I, Melka DC, Sassoubre LM, Boehm AB. 2013. Salmonella enterica Diversity in Central Californian Coastal Waterways. Applied and Environmental Microbiology 79:4199-4209.

Warriner K, Huber A, Namvar A, Fan W, Dunfield K. 2009. Chapter 4 Recent Advances in the Microbial Safety of Fresh Fruits and Vegetables. In: Advances in Food and Nutrition Research, Vol. 57:Academic Press, 155-208.

Weller D, Wiedmann M, Strawn LK. 2015a. Spatial and Temporal Factors Associated with an Increased Prevalence of *Listeria monocytogenes* in Spinach Fields in New York State. Applied and Environmental Microbiology 81:6059-6069.

Weller D, Wiedmann M and Strawn LK. 2015b. Irrigation is Significantly Associated with an Increased Prevalence of *Listeria monocytogenes* in Produce Production Environments in New York State. J Food Prot 78:1132-1141.

Weller DL, Kovac J, Kent DJ, Roof S, Tokman JI, Mudrak E, et al. 2017. *Escherichia coli* transfer from simulated wildlife feces to lettuce during foliar irrigation: A field study in the Northeastern United States. Food Microbiology 68:24-33.

Wilkes G, Edge T, Gannon V, Jokinen C, Lyautey E, Medeiros D, et al. 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res 43:2209-2223.

Winfield MD, Groisman EA. 2003. Role of Nonhost Environments in the Lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environmental Microbiology 69:3687-3694.

Woodward DL, Khakhria R, Johnson WM. 1997. Human salmonellosis associated with exotic pets. Journal of Clinical Microbiology 35:2786-2790.

Wu J, Long SC, Das D and Dorner SM. 2011. Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. J Water Health 9:265-278.

Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, et al. 2009. Prevalence of Antibiotic Resistance in Drinking Water Treatment and Distribution Systems. Applied and Environmental Microbiology 75:5714-5718.

Yachison CA, Yoshida C, Robertson J, Nash JHE, Kruczkiewicz P, Taboada EN, et al. 2017. The Validation and Implications of Using Whole Genome Sequencing as a Replacement for Traditional Serotyping for a National *Salmonella* Reference Laboratory. Frontiers in Microbiology 8:1044.

Yoshida CE, Kruczkiewicz P, Laing CR, Lingohr EJ, Gannon VPJ, Nash JHE, et al. 2016. The *Salmonella* In Silico Typing Resource (SISTR): An Open Web-Accessible Tool for Rapidly Typing and Subtyping Draft *Salmonella* Genome Assemblies. PLoS One 11:e0147101.

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. Applied and Environmental Microbiology 72:5777-5783.

Yu Z, Yang J, Amalfitano S, Yu X, Liu L. 2014a. Effects of water stratification and mixing on microbial community structure in a subtropical deep reservoir. Scientific Reports 4:5821.

Yu Z, Zhou J, Yang J, Yu X, Liu L. 2014b. Vertical distribution of diazotrophic bacterial community associated with temperature and oxygen gradients in a subtropical reservoir. Hydrobiologia 741:69-77.

Zaleski KJ, Josephson KL, Gerba CP, Pepper IL. 2005. Potential Regrowth and Recolonization of Salmonellae and Indicators in Biosolids and Biosolid-Amended Soil. Applied and Environmental Microbiology 71:3701-3708.

Zou W, Lin W-J, Hise KB, Chen H-C, Keys C, Chen JJ. 2012. Prediction System for Rapid Identification of *Salmonella* Serotypes Based on Pulsed-Field Gel Electrophoresis Fingerprints. Journal of Clinical Microbiology 50:1524-1532.