

Factors Influencing *Salmonella* Survival in Agricultural Soils and Internalization through  
Solanaceous Crop Roots

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## ABSTRACT

Solanaceous crops such as peppers and more commonly tomatoes have been linked to *Salmonella* outbreaks that have occurred in the United States. The source of contamination for some of these outbreaks was traced back to the preharvest environment. Sources of contamination at the preharvest level of production often include irrigation water, soil, and the use of biological soil amendments of animal origin (BSAAO). This dissertation aims to (i) determine how factors such as the use of BSAAO's, *Salmonella* strain, soil type, and irrigation influence the survival of *Salmonella* in agricultural soils and (ii) determine the risk of *Salmonella* internalization in to transplanted solanaceous crops (tomatoes and bell peppers) and identify factors that influence internalization through the roots such as mode of contamination, soil contamination level, and root damage. Sandy loam (SL) and clay loam (CL) soils both unamended and amended with poultry litter (PL), irrigated either daily or weekly were inoculated with one of twelve strains of *Salmonella enterica* and monitored for growth and survival until no longer detected. Tomato plants were transplanted into one of three contamination treatments: contaminated soil (A) or irrigation water administered either once (B, single exposure event) or for 7 d following transplantation (C, repeated exposure event). Plants were sampled 1, 2, 3, 7 and 90 d post-transplantation for *Salmonella* internalization. Pepper plant roots were left intact, damaged, or removed and placed in inoculated water (6.2 log CFU/ml). Pepper plants in the soil contamination study, were transplanted into soil inoculated with *Salmonella* at different contamination levels (High, Mid, and Low). Samples were collected 1, 2,

3, and 7d post-transplant. Plants were surface sanitized and enumerated for *Salmonella* internalization by plant section. Strains in soils treated with PL survived significantly ( $P<0.05$ ) longer (56 and 112 days for SL and CL respectively), than *Salmonella* strains in unamended soils. In PL amended SL and CL soils, most strains grew significantly ( $P<0.05$ ) within the first week following inoculation and ranged from 84 to 210 days in the point it was last detected. Strains survival increased significantly ( $P<0.05$ ) in soil irrigated weekly compared to soils irrigated daily, weekly irrigation increased survival by 140 to 224 and 42 to 168 days in SL and CL soils respectively depending on the strain. Root sections in the tomato plants of treatment A had significantly ( $P<0.05$ ) higher recovery of *Salmonella* internalization compared to the other treatments. No tomato fruit sampled were positive for *Salmonella* internalization. Pepper plants with damaged roots had significantly higher ( $P<0.05$ ) *Salmonella* internalization in the stem than plants with intact roots, while plants with no root stalk had significantly higher ( $P<0.05$ ) *Salmonella* internalization in the stem and leaves of plants with intact and damaged roots. Pepper plants exposed to high concentrations of *Salmonella* had significantly more internalization occur in the roots than plants exposed to mid and low concentrations of *Salmonella*. Due to the results of these studies assessing the risk of using PL and irrigation regime should be considered in the *Salmonella* growth and survival in agricultural soils. Though it is unlikely, steps should be considered to limit *Salmonella* contamination of soil and irrigation water and root damage to prevent the internalization of *Salmonella* in tomato and bell pepper plants through intervention measures such as composting, water treatment, and effective transplanting techniques.

# Factors Influencing *Salmonella* Survival in Agricultural Soils and Internalization through Solanaceous Crop Roots

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## GENERAL AUDIENCE ABSTRACT

Produce such as tomatoes and peppers has commonly been associated with *Salmonella* outbreaks in the United States. Fresh produce may be contaminated during the growing period by irrigation water, soil, or manure contaminated with *Salmonella*. This dissertation aims to (i) determine how factors such as poultry litter, *Salmonella* strain, soil type, and irrigation affects the survival of *Salmonella* in agricultural soils and (ii) identify the capability of *Salmonella* uptake through the roots of tomato and bell pepper plants, and identify factors that facilitate the uptake of *Salmonella* such as how the plant is contaminated, the *Salmonella* population level in the soil, and root damage. Sandy loam and clay loam soils untreated and treated with poultry litter, irrigated daily or weekly, were contaminated with one of twelve strains of *Salmonella* and tested for *Salmonella* levels until it was no longer detected in the soils. Tomato plants were planted into one of three treatments: *Salmonella* contaminated soil (A) or irrigated with *Salmonella* contaminated water once after planting (B, single exposure event) or for 7 days following planting (C, repeated exposure event). Tomato plants were tested for *Salmonella* uptake within the first week or 90 days following planting. The roots of pepper plants were either left intact, damaged, or removed and placed into *Salmonella* contaminated water. Pepper plants in the *Salmonella* contaminated soil study were planted into soil containing one of three different *Salmonella* contamination levels (high, mid and low). Plants were tested for *Salmonella* uptake within the first week following planting. Factors such as the use of poultry litter and irrigation influenced the growth and presence of *Salmonella* in both soil types. The uptake of *Salmonella*

of tomato plants in contaminated soil was observed in the roots more than the roots of plants irrigated with contaminated irrigation water. No tomatoes tested were found positive for *Salmonella* internalizing. The uptake of *Salmonella* in the stems and leaves of pepper plants was highly dependent on root damage and the presence of the roots. Pepper plants that were exposed to high levels of *Salmonella* in the soil had more instances of *Salmonella* uptake than plants with lower levels of *Salmonella* in the soil. Based on these results, the use of poultry litter and irrigation should be considered in the potential risk of *Salmonella* contamination during the growing stage of produce. Though the uptake of *Salmonella* in tomato and pepper plants is unlikely, precautions should be taken to limit the potential contamination of soil and irrigation water with *Salmonella* and prevent root damage from occurring to prevent possible uptake of *Salmonella*.

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## Chapter 1: Introduction

Over the last several decades, an increase of produce related foodborne outbreaks has been observed (36). The Centers for Disease Control and Prevention (CDC) reported that nearly half of foodborne illnesses are associated with the consumptions of produce (33). One of the leading causes of bacterial foodborne illnesses is *Salmonella enterica*; in 2016 *S. enterica* outbreaks resulted in 8,172 confirmed cases of foodborne illnesses, 2,233 hospitalizations, and 40 deaths (30). The economic burden from health losses due to foodborne *S. enterica* illnesses in the U.S. is estimated to be \$1.1 billion (35). Between 2015 and 2019 there have been twelve multi-state outbreaks of *S. enterica* associated with fresh or minimally processed produce commodities (7).

Produce may become contaminated at various stages of production. During crop production, irrigation water, and soil (along with the incorporation of biological soil amendments) are likely sources of contamination (5, 34). Irrigation water could easily lead to contamination of the surface of plants and the edible portion of the commodity. Along with surface contamination, internalization of *S. enterica* in tomato plants has been observed when plants have been exposed to contaminated irrigation water (6, 16, 20, 25, 32, 40). Likewise, amended and unamended agricultural soil presents potential surface contamination of fresh produce commodities. *S. enterica* has been shown to survive in agricultural soil for long periods of time depending on factors such as temperature, use of amendments, and moisture (17, 26, 27, 38, 39). *S. enterica* was found capable of surviving in agricultural soil for as long as 336 days following inoculation and presents a concern for potential surface contamination and internalization during crop growth and development (38). Tomatoes have often been implicated

with outbreaks of *Salmonella enterica*, resulting in 17 multistate outbreaks between 1990-2010 (3, 4, 8, 9, 10, 12, 18, 21, 23, 24). Of these outbreaks, five of them were determined that tomatoes were contaminated with *S. enterica* during fruit production (4, 8, 12, 18). These outbreaks call into question the capability of *S. enterica* internalizing in fresh tomatoes during production prior to harvest.

Bacteria that have been found capable of residing internally in plant tissues are known as endophytes. Endophytes are most often bacteria or fungi inhabit living plants internally but most typically extracellularly. Endophytic relationships with plants range from symbionts to neither harming or benefiting plants (15). In recent years, *S. enterica* was found inside tomato, radish, lettuce, and mango plants (13, 14, 31). Due to these findings, *S. enterica* is also considered an endophyte (15). The ability of *S. enterica* to internalize into an edible portion of a plant is exceptionally worrisome due to the fact that surface sanitation and washing steps are ineffective in preventing outbreaks. There are many possible modes for internalization of *S. enterica* in plants, including contamination of seeds, contamination of flowers during fruiting, soil contamination, the use of biological amendments, and contaminated irrigation water (2, 15, 19). Common portals of entry into crop plants could be through lateral roots or wounded roots during seedling development, grafting, or transplanting (1, 22, 37). Endophytic bacteria, including *S. enterica*, have been found to pass through the roots and transport through the vascular system of the plant (11, 28, 29). Little is known about the factors affecting the internalization of *S. enterica* and where it may occur on the farm to fork continuum. Understanding mechanisms and factors that promote the uptake of *S. enterica* during plant development is key in order to prevent further outbreaks due to internalization.

Therefore, the objective of this dissertation is to (i) determine how factors such as serovar, amendments, irrigation regime, and soil type influence the survival of *S. enterica* in agricultural soils; (ii) determine the influence of varying modes of contamination (soil and irrigation water) on the internalization of *S. enterica* in transplanted tomatoes; and (iii) identify the roles root damage and *S. enterica* soil population have on the internalization of *S. enterica* in transplanted bell peppers.

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## Chapter 2: Review of Literature

### Burden of *Salmonella enterica* in fresh produce

*Salmonella enterica* is the leading cause for bacterial foodborne illness in the United States (36). The Centers for Disease Control and Prevention's (CDC) Foodborne Disease Active Surveillance Network (FoodNet) reported 8,172 cases, 2,255 hospitalizations, and 40 deaths related to salmonellosis between 2013-2016 (51). The serovars most commonly related to these outbreaks were Enteritidis (17%), Newport (11%), and Typhimurium (9%). Outbreaks of *Salmonella* in the United States create a staggering economic burden. Health losses alone due to *Salmonella* are estimated to be \$1.1 billion, not including economic losses due to fallout following a foodborne outbreak occurred by the food industry (62). Fresh produce is among the top foods commonly implicated with *Salmonella* outbreaks.

The CDC estimates that 46% of the foodborne outbreaks in the United States are attributed to fresh produce (58). Between 1998-2008, fresh leafy greens and vine-stalk vegetables (i.e. tomatoes) were attributed to 16% and 10% of the hospitalizations due to foodborne outbreaks respectively, vine-stalk vegetables were also responsible for 7% of the deaths related to foodborne outbreaks (58). *Salmonella* is the leading cause of produce related bacterial foodborne outbreaks in the United States (36) and has been linked to over 100 fresh produce related outbreaks between 1998-2012 in the United States (71). Between 2015 and 2019 there have been twelve multi-state outbreaks of *Salmonella* associated with fresh or minimally processed produce (11). Along with the United States, *Salmonella* is responsible for the majority of produce related bacterial foodborne outbreaks in the European Union (10). Fresh produce contamination may occur during production and post-harvest. Of the outbreaks associated with

fresh produce between 2004-2012, tomatoes were the commodity most commonly implicated with *Salmonella* (10). Outbreaks of *Salmonella* have commonly been linked to fresh tomatoes and will be discussed further.

#### Tomato associated outbreaks of *Salmonella enterica*

Tomato associated *Salmonella* outbreaks have been a problem for several years. These outbreaks vary in location, number of cases, serovar, and the point of contamination in tomato production (Table 1.1). In 1990 and 1993, two outbreaks of *Salmonella* Javiana and Montevideo, respectively, were associated with a tomato packing house in South Carolina that resulted in 276 cases (37). Between 1998-1999, raw tomatoes were implicated in a multistate outbreak of *Salmonella* Baildon that resulted in 86 cases and 3 deaths traced back to growers in Florida. Further investigation determined that contamination occurred during preharvest from contaminated irrigation water that contacted fruit (16). An outbreak in 2000 of *Salmonella* Thompson in fresh tomatoes resulted in 42 cases of salmonellosis (36). *Salmonella* Newport has been associated with 6 tomato outbreaks in 2002, 2005, 2006, 2007, and twice in 2010, resulting in a total of 912 cases. Of the six outbreaks, five were traced back to the Eastern Shore of Virginia and one in Florida, three of which traced back the irrigation water as a potential point of contamination at preharvest (4, 5, 12, 26). In 2002, tomatoes were again implicated with an outbreak of *Salmonella* Javiana resulting in 141 cases. Three outbreaks of *Salmonella* in tomatoes occurred in 2004 involving multiple serovars including Javiana, Typhimurium, and Braenderup resulting in 561 cases of salmonellosis (14, 33). Between 2005-2006 three more outbreaks of *Salmonella*, not including the previously mentioned *Salmonella* Newport outbreaks, occurred involving serovars Berta, Braenderup, and Typhimurium, resulting in 288 cases (12,

25). The tomatoes involved in the Braenderup outbreak were traced back to a grower in Florida and contamination was determined to have occurred during production after isolates were collected from irrigation ditches and animal feces around the tomato field. (12). These outbreaks show the potential for contamination to occur on tomato during production.

**Table 1.1** *Salmonella enterica* outbreaks due to contaminated tomatoes between 1990-2010

<b>Serovar</b>	<b>Year</b>	<b>Point of Contamination</b>	<b># of Cases</b>	<b>Reference</b>
Javiana	1990	Packing House	176	(37)
Montevideo	1993	Packing House	100	(37)
Baildon	1998-1999	Preharvest	86	(16)
Thompson	2000	Unknown	42	(36)
Javiana	2002	Unknown	141	(13)
Newport	2002	Preharvest	510	(25)
Multiple*	2004	Unknown	429	(14)
Braenderup	2004	Unknown	125	(14, 33)
Javiana	2004	Unknown	7	(14)
Newport	2005	Preharvest	72	(12, 25)
Braenderup	2005	Preharvest	82	(12)
Newport	2006	Unknown	115	(12)
Typhimurium	2006	Unknown	190	(12)
Berta	2006	Unknown	16	(36)
Newport	2007	Unknown	65	(5)
Newport	2010	Unknown	99	(4)
Newport	2010	Preharvest	51	(5)

\*Javiana (383), Typhimurium (27), Anatum (5), Thompson (4), Muenchen (4), and Group D untypable (6)

#### The role of *Salmonella enterica* as an endophytic bacterium

Bacteria capable of residing internally in the tissue of plants while providing a beneficial relationship with the plant or not providing any benefit or doing any apparent harm are called endophytes (20). There are various portals of entry for endophytes, but entry commonly occurs in the stomata, hydathodes, lateral roots, wounds and stem scars of the plants (20). It is common for endophytic bacteria to infiltrate plant tissue by the root hairs or junctions between the root hairs and epidermal cells (35, 66). Once internalized in the roots, transport through the xylem

may occur (47). Plants may experience stresses caused by drought, extreme temperatures, and disease making the plant more susceptible to infiltration of endophytic bacteria (50). Some human bacterial pathogens can be defined as endophytes and are conditioned to survive in the germinated seeds, roots, and leaves of plants.

*Salmonella* can reside for long periods of time inside various plant tissues making it an endophyte (18, 19, 53). In the absence of microbial competitors, *Salmonella* was found capable of internalizing and moving along the xylem of *Arabidopsis* that resulted in contamination of the whole plant (15). From the study performed by Cooley et al., it was determined that nonmotile *Salmonella* mutants were significantly less capable of invasion and unable to move along the plant, suggesting that internalization is based on flagellar motility and on organism activity (15). The capabilities of *Salmonella* internalization in tomato plants will be discussed further, as well as how *Salmonella* may be introduced to the plants in order to internalize.

#### Internalization of *Salmonella enterica* in tomato plants

Several studies have examined the internalization of *Salmonella enterica* in tomato plants through a variation of entry points, serovars, inoculum levels, and growth conditions. It has been identified that *Salmonella enterica* is capable of internalizing into the fruit through inoculated flowers (30, 63, 73). Guo et al. found that of the flowers inoculated with a cocktail of *Salmonella* serovars Montevideo, Michigan, Poona, Hartford, and Enteritidis, 25% of the fruit was found positive with internalized *Salmonella* (30). In a study performed by Shi et al., flowers were inoculated with  $10^7$  CFU/ml culture containing one of the following serovars; Javiana 5913, Javiana 6027, Montevideo, Newport, Enteritidis, Senftenberg, Typhimurium, Hadar, Infantis, and Dublin (63). Tomatoes were sampled and determined for presence/absence using Rappaport-

Vassiliadis agar and streaked on to xylose lysine deoxycholate agar (XLD). Serovars Montevideo (90%), Hadar (56%), Newport (44%), and Javiana 6027 (40%) had the highest percent of internalization from the positive batches. Similarly, Zheng et al. found that Javiana, Montevideo, and Newport along with Saintpaul were capable of internalizing in tomato fruit when the flower was inoculated with a 5 serovar *Salmonella* cocktail (73). Interestingly, both studies showed that *Salmonella* serovar Typhimurium was unable to internalize in tomato fruit from the inoculated flowers (63, 73).

*Salmonella enterica* was also found capable of internalizing into inoculated tomato plant leaves, which in some cases lead to the internalization in the fruit. Gu et al. found that *Salmonella* Typhimurium was able to internalize and persist for several days in the leaves of various tomato cultivars but was most successful in internalizing in the “Ailsa Craig” cultivar (26). Similarly, Zheng et al. found that a cocktail of *Salmonella* serovars was capable of colonizing tomato plants when wounded leaves were spot inoculated (73). In another study performed by Gu et al., infiltration of *Salmonella* Typhimurium occurred in leaves of tomato plants grown in conventional and organic soils and transferred into the fruit (27). The movement of *Salmonella* Typhimurium when internalized in the tomato plant through the leaves was confirmed, as Gu et al. determined that uninoculated leaves and fruit adjacent to dip inoculated leaves were found positive for *Salmonella* internalization (28).

Along with leaves, *Salmonella enterica* was found capable of moving along the vascular tissue of the plant and into the fruit (28, 30). When the stems of tomato plants were injected with a cocktail of *Salmonella* serovars Montevideo, Michigan, Poona, Hartford, and Enteritidis, internalization was found in 43% of tomatoes when the injection occurred before flower set and

40% of tomatoes when the injection occurred after flower set (30). After injection of *Salmonella* Typhimurium into the peduncle of the plant, *Salmonella* was able to move into the fruit and grow approximately 6 log CFU/g over the period of two weeks (28). These results show that *Salmonella enterica* is more than capable of moving along the xylem from the stem but does not answer if *Salmonella* is capable of moving from the roots into the fruit of tomato plants.

Several studies have explored the capabilities of *Salmonella* to internalize in tomato plants when exposed to the roots in various modes such as in a hydroponic system, irrigation water, or in soil. When *Salmonella* was incorporated into irrigation water, several studies identified uptake occurring only through plant roots with no further movement up the stem (7, 54). Contrary to these results, when *Salmonella* was incorporated into hydroponic solution, internalization occurred in the roots, stems, and leaves of tomato plants (31). When tomato seeds were inoculated with *Salmonella*, internalization occurred in roots and stems of seedlings (48). Likewise, when *Salmonella* was exposed to the root section of tomato plants transplanted into soil, several studies found internalization occurred in the roots, stems, leaves, and in some cases even the fruit (38, 60, 73). In a study performed by Hintz et al., after plants were irrigated with water inoculated with *Salmonella* Newport, 6% of the fruit tested positive for internalization after enrichment (38). Similarly, Zheng et al. found internalization of *Salmonella* in 5.5% of tomatoes tested (73). In comparison, Pollard et al. discovered tomato plants inoculated with *Salmonella* Montevideo and *Ralstonia solanacearum* via a root dip method had more *Salmonella* internalization when the two organisms were administered in irrigation water together (3.5%) than if only *Salmonella* was administered in the irrigation water (1.7%) (60). In a study performed by Karmakar et al., internalization of *Salmonella* through the roots was determined to

be through the lateral roots of the plant, and that internalization of the pathogen is dependent on the number of lateral roots (44). In comparison *Listeria monocytogenes* and *E. coli* O157:H7 only internalized in roots of tomato plants irrigated with inoculated water (23). Though the occurrence may be rare, previous studies have found that *Salmonella* is capable of internalizing in tomato fruit while on the vine through the roots, though this may be dependent on aspects such as inoculum level, how the inoculum is administered, plant type, stage of plant development or the serovars used.

#### Modes contamination of *Salmonella enterica* in tomatoes at the pre and postharvest stages

Due to the inability to control every potential risk faced during tomato growth and harvesting, various modes of contamination exist at different production stages. Effective targets have been determined to reduce contamination along the different stages of growing fresh market tomatoes. Tomato fruit contamination can occur during production or postharvest handling. Each type of contamination will be discussed further.

#### **Preharvest**

Tomatoes are either grown in the field, where more variation of environmental conditions exist, or in a greenhouse, where conditions are more controlled. During field production, irrigation water and soil (including the use of biological soil amendments) are the most likely sources of contamination of fresh market tomatoes during production (2, 3, 6, 59). During the production of greenhouse tomatoes using advanced technologies, soilless media is often used instead with hydroponic solution supplying both water and nutrients. Some organic greenhouse growers use certified organic solutions that utilize compost tea or liquid formulations of organic material. Animal or pest intrusion (domestic or wild), and/or worker hygiene have been

identified as potential surface contaminants in both field and greenhouse production (6, 36, 40, 59, 61). Contamination sources for internalization of pathogens were determined to be by spray and drip irrigation as well as manure application (20).

Contaminated irrigation water is a major concern with potential transmission to tomato plants and fruit, especially if the contaminated water is used for the application of pesticides, herbicides and overhead irrigation. In studies conducted by Guan et al., *Salmonella* was capable of surviving in and being transferred onto the plants and fruit during application of pesticides, and then persisting on the surface of the plant and fruit for 56 days following application (29).

Unprotected surface irrigation water may be contaminated by livestock or wildlife.

Environmental sampling studies performed in New York and the Eastern shore of Virginia isolated *Salmonella* from surface water source used for the irrigation of produce (4, 64, 65). It has been determined that irrigation water that was naturally contaminated with foodborne pathogens could transfer and contaminate edible portions of vegetable crops (49, 52). In a study performed by Jablasone et al., irrigation water inoculated with *Salmonella* was administered to tomato plants via a drip system which resulted in the contamination of plant foliage and *Salmonella* populations were able to survive relatively unchanged in the soil for five weeks thus providing potential for contamination if the fruit came in contact with the soil or splashing occurred during a heavy rain (43).

Many other studies have found that *Salmonella* is more than capable of surviving for long periods of time in both treated and untreated soils used for produce production (1, 9, 17, 24, 32, 34, 39, 46, 56, 57, 68, 72). Arthurson et al. found that *Salmonella* serovar Weltevreden was able to survive in clay loam soil treated with cattle manure for up to 21 days with populations

remaining relatively unchanged (1). *Salmonella* serovar Thompson was exposed to wet and dry conditions of the phyllosphere and was found to grow in the soil during wet conditions as opposed to the dry conditions (9). In a study performed by Danyluk et al., *Salmonella* serovar enteritidis was detected by enrichment in a clay loam soil 180 days following inoculation (17). Guo et al. found that a *Salmonella* cocktail was capable of surviving for 45 days in moist topsoil, and *Salmonella* populations had a 2.5 log CFU/tomato increase on tomatoes that came into contact with the soil (32). In a study performed by Ongeng et al. *Salmonella* Typhimurium survived longer in soil amended with cattle manure than in the manure alone, with populations surviving for up to 14 weeks (56). Underthun et al. found that a five-strain cocktail of *Salmonella* (Anatum, Braenderup, Javiana, Montevideo, and Newport) inoculated Candler sand and Orangeburg sandy loam soils was able to survive for up to 336 and 329 days, respectively, based on the storage temperature and volumetric water content (68). In a study conducted by Holley et al., soils treated with manure generally had higher populations of *Salmonella* than untreated soils (39). The ability for *Salmonella* to survive for long periods of time in treated and untreated agricultural soils raises a concern of potential contamination of vegetables during production. The incorporation of biological soil amendments increases risk of plant surface contamination. Studies have shown the plants can become contaminated when grown in soil inoculated with cattle manure and chicken litter (22, 41, 42, 45, 55, 57).

## **Postharvest**

During postharvest handling, tomatoes can be contaminated from reusable containers, worker contact, pests in the packinghouse, and/or vehicles; however, the most common risk is wash water (36, 40). Following harvest, tomatoes that are grown in the field usually undergo a

washing process to remove any dirt or debris on the surface. This washing step may introduce potential contaminants to the tomatoes if the water isn't properly sanitized. Internalization of the pathogens into the fruit may occur in the stem scar or other wounds that may have occurred during growing or harvest (20). Internalization may also occur if *Salmonella* is present in the wash water or on the surface of the fruit and the fruit is washed in water that is colder than the fruit or at a depth that would create hydrostatic pressure (70). Turner et al., determined that the time a tomato is submerged in the water influences the internalization of *Salmonella* more than the temperature differential between the fruit and the wash water (67). Another study found that immersion time, depth of immersion, and temperature differential all significantly influenced internalization of *Salmonella* (74).

Once on the surface or internalized into the fruit, *Salmonella* may survive and even grow over time depending on storage conditions. Shi et al., determined that growth of *Salmonella* on the surface and in the pulp of tomatoes was promoted by high temperatures (25 °C) and high relative humidity (95%), though these findings were serovar dependent (63). In another study, *Salmonella* was able to grow in the stem scar and pulp of a variety of tomatoes stored at 12 and 21 °C (8). Contrary to these results, in a study performed by Zhou et al., temperature did not influence the population changes of *Salmonella* internalized in tomatoes, but rather serovar had a significant contribution to survival and growth (74). Serovars Hadar, Montevideo, Newport, and Thompson were found capable of growing in tomatoes following harvest (63, 74).

#### Food safety programs specific for tomatoes

In order to identify and prevent produce contamination, such as the Produce Safety Alliance (PSA) and the United States Department of Agriculture's (USDA) Agricultural

Marketing Service's Good Agricultural Practices (GAPs), exist to train growers about risky behaviors and ensure safe practices are implemented. Further guidelines for high-risk produce items are also given in order to ensure commodity specific risky behaviors are avoided or controls are implemented. Along these lines, the U.S. Food & Drug Administration (FDA) has developed a guide to minimize the microbial food safety hazards for tomatoes; this guide includes refraining from using raw animal manure, verifying the time and temperature of the composting processes, and ensuring water used for crop protection sprays is not contaminated or has been treated (69).

Along with guidance from the federal government, some states have mandatory programs for safe tomato growing and harvesting practices. In Florida the Tomato Good Agricultural Practices (T-Gap) program is mandatory and all growers and packers are subject to mandatory state inspections and audits by the Florida Department of Agriculture and Consumer Services (21). This program requires growers, packers, repackers and workers to complete annual education trainings, as well as extensive requirements for areas such as irrigation water, worker hygiene, crop production practices (including manure requirements), harvesting, equipment, debris removal, removal of injured fruit, and record keeping (21). The water requirements include that water used for any foliar application at the time of harvest must meet the same microbial standards for potable water, and water being used for irrigation is not contaminated with human or animal feces and while meeting the same standards as recreational water (21). Along with water standards, this program only allows the use of manure that has been composted using the Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables, with documentation of the date, method, and application of the compost (21).

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### Chapter 3: Soil-type, Irrigation Regime, and Poultry Litter Influence *Salmonella* Strain Survival in Agricultural Soils

*Formatted for Frontiers in Microbiology*

#### ABSTRACT

The use of untreated biological soil amendments of animal origin (BSAAO) has been identified as a *Salmonella* source in produce farm environments. Data on factors influencing *Salmonella* dynamics in amended soils are therefore needed. Thus, the objectives here were to (i) compare die-off between 12 *Salmonella* strains following inoculation in amended soil, and (ii) characterize effect the effect of soil type (sandy-loam vs clay-loam), irrigation (daily vs weekly), and amendment (poultry litter vs non-amended) on *Salmonella* survival and die-off. Three greenhouse trials were performed using a randomized complete block design. Each strain (~4 log CFU/g) was homogenized with amended or non-amended sandy- or clay-loam soil. *Salmonella* levels were enumerated in 25 g samples 0, 0.167 (4 h), 1, 2, 4, 7, 10, 14, 21, 28, 56, 84, 112, 168, 210, 252, and 336 d post-inoculation, or until two consecutive samples were negative by enrichment. Regression was used to characterize associations between strain, soil-type, irrigation, and (i) time to last detect (survival) and (ii) concentration at each time point (die-off rate). Similar effects of strain, irrigation, soil type and amendment were found using the survival and die-off models. Strain explained up to 18% of the variance in survival, and up to 19% of variance in die-off rate. On average *Salmonella* survived for 129 d in amended soils, however *Salmonella* survived, on average, 30 days longer in sandy-loam soils than clay-loam soils [95% Confidence Interval (CI)=-45, -15;  $P<0.001$ ]. *Salmonella* also survived, on average, 128 d longer when irrigated weekly, compared to daily (CI=101, 154;  $P<0.001$ ), and 89 d longer in amended

soils, then non-amended soils (CI=61, 116;  $P<0.001$ ). Interestingly, when strain-specific associations were investigated using regression trees, we found that although most strains survived longer in clay-loam soil, *S. Javiana* and *S. Saintpaul* survived longer in sandy-loam soil. Overall, this study provides insight into *Salmonella* survival following contamination of field soils by BSAAO. Specifically, *Salmonella* survival is affected by soil characteristics and management practices, and that the effect of these factors may be strain specific. Such data can assist in risk assessment and strain selection for use in challenge and validation studies.

## INTRODUCTION

Past reports estimate that *Salmonella* is the leading cause of bacterial foodborne disease outbreaks linked to fresh produce in the United States (US) and Europe (Hanning et al., 2009; Callejón et al., 2015). Fruits and vegetables are particularly problematic as an etiological vehicle due to the lack of a kill step between harvest and consumption that would eliminate or reduce pathogen loads. Thus, there is particular interest in preventing *Salmonella* contamination of fresh produce, including in the pre-harvest environment. As illustrated by past *Salmonella* outbreaks in the Delmarva region (Greene et al., 2008; Hanning et al., 2009; Painter et al., 2013; Bell et al., 2015; CDC, 2016; Truitt et al., 2018), there are multiple mechanisms whereby produce can be contaminated in the farm environment. In response to the aforementioned outbreaks, multiple studies investigated potential sources of the outbreak serovar in the Delmarva, and identified wildlife (Gruszynski et al., 2014), irrigation water (Greene et al., 2008; Hanning et al., 2009), and the use of biological soil amendments of animal origin (BSAAO) as potential *Salmonella* sources (CDC, 2016; Gu et al., 2019). Moreover, studies in other produce-growing regions (e.g., California, New York, Florida) have also detected *Salmonella* in agricultural field soils (Gorski

et al., 2011; Strawn et al., 2013a, 2013b, 2014; Bell et al., 2015), irrigation water (McEgan et al., 2013; Cooley et al., 2014; Weller et al., 2015, 2020), and BSAAOs (Gu et al., 2019). As such, preventing pre-harvest produce contamination is a priority for industry and public health professionals.

Since the pre-harvest environment represents a key route for *Salmonella* contamination of fresh produce, understanding pathogen ecology in pre-harvest environments, including factors affecting pathogen survival and growth, is key to developing effective food safety risk mitigation strategies. For example, past studies (Chandler and Craven, 1980; Danyluk M.D. et al., 2007; Underthun et al., 2017; Jechalke et al., 2019) have suggested that *Salmonella* can survive for long periods in soils and survival was dependent on soil characteristics (e.g., moisture, composition, temperature). Specifically, the use of BSAAOs appears to facilitate *Salmonella* survival (Baloda et al., 2001; Holley et al., 2006; You et al., 2006; Nyberg et al., 2010; Ongeng et al., 2011; Hruby et al., 2018; Shah et al., 2019). For example, a study conducted in Iowa (2018) detected *Salmonella* in soil samples collected from plots amended with poultry litter approximately one year after BSAAO application. Similarly, a study (You et al., 2006) recovered *Salmonella* from bovine manure-amended soils 332 d after application in the laboratory (controlled temperature and moisture). These findings are concerning from a food safety standpoint because *Salmonella* in soils contaminated by BSAAO can transfer to the harvestable portion of crops (Natvig et al., 2002; Islam et al., 2004a, 2004b; Franz et al., 2005; Kenney et al., 2006; Oni et al., 2015; Kumar et al., 2017). Cessation of BSAAO application to meet food safety concerns is impractical since BSAAOs, such as poultry litter, are economical, organic fertilizers whose application promotes soil health and fertility (Watts et al., 2010; Gu et

al., 2019). As such, additional research is needed to better understand *Salmonella* dynamics following BSAAO amendment and provide growers with specific guidance on how to best manage food safety hazards in BSAAOs.

Recognizing the food safety risks associated with as well as the economic importance of BSAAOs, stakeholders have developed recommendations on intervals between application of untreated BSAAO and harvest. For example, the United States Department of Agriculture (USDA) National Organic Program (NOP) recommends intervals of >120 d between manure application and harvest if the edible/harvestable portion of the produce has direct contact with the soil (USDA, 2015; USDA 2020). However, it is unclear (i) if existing data support NOP guidelines and (ii) if a one-size-fits all guideline is appropriate for all commodities, management practices (e.g., type of BSAAO used, irrigation regime), produce-growing regions, and even fields within a single operation. Indeed, past studies have shown that soil characteristics, environmental and laboratory conditions (e.g., temperature), and amendment type can all affect *Salmonella* survival in soil (You et al., 2006; Semenov et al., 2009; Ramos et al., 2019). Similarly, past studies (Strawn et al., 2014; Weller et al., 2015; Sharma et al., 2020) indicates that factors that vary between produce-growing regions, between farms within the same region, and between fields on the same farm can affect pathogen dynamics in farm environments. Additionally, recent studies (Andino and Hanning, 2015; Harrand et al., 2019) have shown that differences between pathogen strains can result in substantially different responses to environmental stresses. As such, the study reported here was performed to (i) investigate survival and die-off between 12 *Salmonella* strains following inoculation in poultry litter amended soil,

and (ii) characterize the effect of soil type (sandy-loam vs clay-loam), irrigation regime (daily vs weekly), and amendment (poultry litter vs non-amended) on *Salmonella* survival and die-off.

## **MATERIALS AND METHODS**

A greenhouse experiment consisting of three separate trials was performed at the Virginia Tech Eastern Shore Agricultural Research and Extension Center (Painter, VA, USA).

Experiment design elements and parameters were adapted, when applicable, from a previously published framework for developing research protocols using the good, better, and best experimental designs for model system trials (Harris et al., 2013). Trials were arranged in a randomized complete block design with experimental units (i.e. pots) organized on wire tables, no closer than 15.24 cm apart. Each trial consisted of one pot per strain and soil type (12 pots for sandy loam and 12 pots for clay loam) that received daily irrigation, one pot per three selected strains and soil type (3 pots for sandy loam and 3 pots for clay loam) that received weekly irrigation; all of these pots were amended with poultry litter. Additionally, two pots, one with poultry litter amended soil and one with un-amended soil, per trial were inoculated with one *Salmonella* strain; both pots received daily irrigation. Each trial consisted of 32 pots (experimental units); excluding control pots (n=6 each trial), which are discussed below (for a total of n=96 experimental units and 18 controls). Greenhouse conditions were maintained to simulate the spring growing season in Virginia (Mid-Atlantic region, US). Temperature and relative humidity conditions (HOBO Micro Station Data Loggers, Onset, Bourne, MA, USA) within the greenhouse were monitored over the duration of all experiments and recorded at every sampling time-point (see **Figure S3.1**).

### **Soils and Poultry Litter**

Two soil types were used: sandy-loam and clay-loam. Sandy-loam soil was obtained from a farm on the Eastern Shore of Virginia (Painter, VA, USA), while clay-loam soil was obtained from a farm in mainland Virginia (Petersburg, VA, USA). Soil was obtained for each experiment from 0-20 cm layer of agricultural fields (used to grow vegetables), sieved (to remove rocks, debris and large aggregates), and air-dried for 7 d. Poultry litter was obtained from a local chicken operation on the Eastern Shore of Virginia (Melfa, VA, USA) the morning before each experiment. Physical/chemical properties, such as pH, moisture, total carbon, total nitrogen, total Kjeldahl nitrogen, ammonium nitrogen, and phosphorus, of the sandy-loam, clay-loam, and poultry litter were determined by standard testing methods, and are reported in **Tables S3.1** and **S3.2** (Waypoint Analytical; Richmond, VA, USA). Soil was weighed into 1 kg portions and added to new 1.89 L plastic nursery pots (nursery pots were sprayed with 70% ethanol and dried 24 h prior to use in a biosafety cabinet). Poultry litter was added to soil based on a rate of 8.9 Mg/ha (or 4 tons per acre), which is used commercially. Sterilized distilled water was added to each pot to bring the soil moisture to levels that typically observed in agricultural fields (approximately 12-15% sandy-loam and 22-25% clay-loam).

### ***Salmonella* Strains and Inoculum Preparation**

Twelve *Salmonella enterica* strains were used in this study (**Table 3.1**), representing a diverse range of serovars and sources (e.g., environmental, clinical, food, animal), including Braenderup (CFSAN038461, cucumber), Enteritidis (SAL 1985, romaine lettuce), 4, 12:i:- (RM17301, environmental), Javiana (CFSAN003355, environmental), Meleagridis (MJRL, cattle), Montevideo (SAL 2345, iceberg lettuce), Muenchen (LJH0592, orange juice outbreak), Newport-E (SAL 2133, environmental), Newport-F (CFSAN003880, almond), Paratyphi B

(CFSAN0086, environmental), Poona (MDD237, clinical-cantaloupe linked), and Saintpaul (SAL 2354, clinical-jalapeno pepper linked). All strains were adapted to nalidixic acid (NA; Fisher Scientific, Fair Lawn, NJ, USA) using a stepwise procedure as previously described (Parnell et al., 2005). Strains were adapted to grow in the presence of an antibiotic, as previously described (You et al., 2006; Semenov et al., 2009; Underthun et al., 2017; Wang et al., 2018), to assist in distinguishing inoculum from background microflora. Briefly, strains (each 12 NA adapted *Salmonella*) were cultured from frozen stock (-80°C) on tryptic soy agar plates (TSA; Difco, BD, Sparks, MD, USA) supplemented with 50 ug/mL NA and incubated at 37°C for 24 h immediately prior to each experiment. The inoculum was prepared using a modified version of a previously described protocol (Sharma et al., 2016). Briefly, three to five well isolated colonies (pure culture) of each strain were inoculated into 200 mL of tryptic soy broth (TSB; Difco, BD) with 50 ug/mL NA, and incubated at 37°C for 18 h with agitation (140 rpm). To achieve a target starting inoculum of 6 log CFU/mL, a 10 mL aliquot of each 18 h culture was added to 990 mL of 0.1% peptone water (Difco, BD). To confirm the target starting inoculum, serial dilutions in 0.1% peptone water were plated onto TSA and xylose lysine tergitol (XLT-4; Difco, BD) supplemented with 50 ug/mL NA, incubated at 37°C for 24 h, colonies were then enumerated, and log CFU/mL calculated.

### **Inoculation Protocol**

In a sterile bag (Fisher Scientific), 100 mL of each *Salmonella* strain inoculum were mixed with 1 kg of poultry litter amended soil and sterile distilled water, where it was thoroughly homogenized using a 30 s shake, 30 s rub, 30 s shake procedure, repeated for five min. Sterile distilled water was added to achieve the targeted soil moisture level (approximately 12.5% and

22% for sandy-loam and clay-loam soils, respectively). Preliminary experiments to test the inoculation protocol showed that amended soil in pots had a uniform mixture of each *Salmonella* strain throughout (data not shown; available upon request). The resulting mixture was returned to the pot, and each pot transferred to the greenhouse. The target inoculum in each pot with amended soil was approximately 4 log CFU/g. Six control pots per trial: un-inoculated PL amended sandy- and clay-loam soil irrigated daily and weekly (4) and un-inoculated non-amended sandy- and clay-loam soils irrigated daily (2), were inoculated with sterile distilled water.

### **Sampling and Recovery of *Salmonella***

Twenty-five g soil samples were collected from each pot, and the *Salmonella* concentration enumerated at 0, 0.167 (4 h), 1, 2, 4, 7, 10, 14, 21, 28, 56, 84, 112, 168, 210, 252, and 336 d post-inoculation, or until *Salmonella* could no longer be detected after two consecutive sample collections. Five sub-samples (representing a 25 g sample) were collected from each pot (i.e., 25 g total) and combined with 225 mL of buffered peptone water (BPW; Difco, BD) in a sterile, filtered Whirl-pak bag (Nasco, Modesto, CA, USA). Each sample bag was shaken and hand massaged vigorously for 60 s. A standard direct plating method (Underthun et al., 2017) was used until counts neared the limit of detection (LOD = 1 log CFU/g). Briefly, 1 mL of each homogenate (25 g sample and 225 mL BPW) was serially diluted in 9 mL of 0.1% peptone water, and 0.1 mL was plated on XLT-4 supplemented with 50 ug/mL nalidixic acid, to enumerate each *Salmonella* strain population. Additionally, 1 mL (0.25 mL aliquoted on each of four plates) was plated from the sample bag to reach a LOD of 1 log CFU/g. All agar plates were incubated at 37°C for 24 h. Once *Salmonella* counts neared the LOD for the direct plating

method, a modified *Salmonella* MPN method (Gartley et al., 2018; Jay-Russell et al., 2018; FDA, 2019) was used to increase the LOD to 0.25 (-0.6 log) MPN/g. Briefly, 5 mL of the sample homogenate was transferred into the first column of a 48 well reservoir (8 by 6 well reservoir; with subsequent columns filled with 4.5 mL of BPW). Each sample was serially diluted (1:10) in each subsequent column (two through six) and incubated at 37°C for 20 h with 50 rpm, shaking. A 50 uL aliquot from each well was then transferred into the corresponding wells of a 48 well reservoir of Rappaport Vassiliadis broth (RV; Difco, BD; 5 mL of RV in each well), and incubated at 42°C for 48 h with 50 rpm, shaking. After incubation, 10 uL from each well was channel streaked onto XLT-4 supplemented with 50 ug/mL nalidixic acid, and incubated at 37°C for up to 48 h. The number of *Salmonella* positive streaks was recorded out of eight possible *Salmonella* positive streaks (presumptive *Salmonella* positives produce black streaks with a pink halos). Presumptive *Salmonella* positive streaks were re-streaked onto TSA agar plates, incubated at 37°C for 24 h, and up to four well isolated colonies per streak were confirmed by PCR amplification of the *invA* gene (McEgan et al., 2013; Strawn et al., 2013a). Confirmed *Salmonella* positive streaks were counted, and MPN values calculated (<http://standards.iso.org/iso/7218/>). Sample homogenate was also enriched concomitantly with the MPN enumeration using standard FDA BAM methods based on a 25 g sample (FDA, 2019).

### **Irrigation Regime**

The two irrigation regimes used in this study were implemented either by tracking weight changes in each pot and adjusting for the water loss by addition of sterile distilled water on a daily or weekly basis to achieve the targeted soil moisture level (12.5% for SL soil and 22% for CL soil), as previously described by Underthun et al., 2018.

## Statistical Analysis

All model-based analyses were performed in R version 3.3.1 or 3.3.5 (R Foundation for Statistical Computing, Vienna, Austria). For all analyses, the data were divided into three subsets: (i) pots amended with poultry litter that were inoculated with *S. Braenderup*, *S. Enteritidis*, *S. 4, 12:i:-*, *S. Javiana*, *S. Meleagridis*, *S. Montevideo*, *S. Muenchen*, *S. Newport-E*, *S. Newport-F*, *S. Paratyphi B*, *S. Poona*, or *S. Saintpaul*, and received daily irrigation (“the daily dataset”; N=72), (ii) pots amended with poultry litter that were inoculated with *S. Braenderup*, *S. Newport-F*, or *S. Meleagridis*, since these were the three strains that received daily and weekly irrigation (“the irrigation dataset”; N=18), and (iii) pots that were inoculated with *S. Newport-E*, since this was the only strain that was inoculated into pots with and without poultry litter amendment (“the amendment dataset”; N=6).

Effect of Soil-Type, Irrigation, and Amendment on *Salmonella* Survival and Die-off. The daily dataset was used to quantify the impact of soil-type on die-off rate while controlling for strain. The irrigation dataset was used to quantify the impact of irrigation regime on die-off rate while controlling for strain, and the amendment dataset was used to quantify the impact of amendment on die-off rate while controlling for strain. To perform these analyses, two sets of general linear models were developed. The outcome of the two models sets were (i) time to last detection of *Salmonella*, which was used to examine the effect of each factor (soil-type, strain, irrigation regime, and soil amendment) on *Salmonella* survival and (ii) concentration of *Salmonella* at each time point (log CFU or MPN/g), which was used to examine effect of each factor on die-off rate. Depending on the dataset, a fixed effect of irrigation regime (weekly or daily; irrigation dataset) or amendment (poultry litter-amended or non-amended; amendment

dataset) was also included. In the models where the outcome was *Salmonella* concentration at each time point, time since inoculation was also included as a fixed effect. All general linear mixed models included soil-type (sandy- or clay-loam) and trial (1, 2 or 3) as fixed effects, and strain as a random effect.

Effect of Strain *Salmonella* Survival and Die-off. The daily dataset was used to compare the die-off rate for each of the 12 strains in poultry litter amended soils. Using the LmList function in the *lme4* R package separate linear models were built for each *Salmonella* strain. The outcome was *Salmonella* concentration, and the fixed effects were time since inoculation, trial, and soil type. Die-off rates between strains were compared visually using the 95% confidence intervals (CI) for the effect estimate of time since inoculation. If 95% CI did not overlap, it was concluded that the die-off rates between the two strains were significantly different.

Effect of Interactions between Soil-Type, Strain Irrigation and Amendment on *Salmonella* Survival. To identify specific combinations of soil-type, irrigation regime, soil amendment, and strain associated with decreased or increased *Salmonella* survival, a conditional inference tree (cTree) was built using the full dataset and the partykit package. Since the smallest number of pots included in a given treatment were 3, the minbucket parameter was set to 6 to prevent overfitting; the mincriterion parameter was also set to 0.95 to prevent overfitting. Separate trees were also built using the same parameters but using the individual datasets (as opposed to the full dataset), to see if there were additional interactions, not evident when the full dataset was used.

## **RESULTS AND DISCUSSION**

No significant difference was observed between the three trials ( $P>0.05$ ) for any of the analyses. The regression tree analysis showed that strain, soil-type, irrigation regime, and amendment all significantly influenced the survival of *Salmonella* in agricultural soils (**Figure 3.1**). The findings of the regression tree analysis are supported by the results of the general linear mixed models, which also showed that soil-type, irrigation regime, and amendment significantly impacted *Salmonella* survival time (**Table 3.2**) as well as die-off rate (**Table 3.3**) ( $P\leq 0.05$ ).

**Survival and die-off rates in amended soils receiving daily irrigation was significantly associated with *Salmonella* strain.** Across the 96 pots, the total time that *Salmonella* survived ranged between 84 and 210 d (Mean=144 d; **Figure 3.2**). In poultry litter amended sandy-loam soil pots, the concentration of all strains, except *S. Poona* increased between inoculation and 7 d post-inoculation (dpi) and remained elevated until 14 dpi (**Table S3.3**). On average, *Salmonella* populations started to decrease 14 dpi (**Table S3.3**). Conversely, in amended clay-loam soil, all strains observed a 0.5 to 1.5 log CFU or MPN/g reduction 4 h dpi (**Table S3.4**). While *S. Saintpaul* was the only strain to sort into the node for longer survival (average 168 d) in both sandy- and clay-loam amended soils, *S. Javiana*, and *S. 4,12:i-*, *S. Braenderup*, *S. Enteritidis*, *S. Montevideo*, *S. Newport-F*, *S. Newport-E*, *S. Paratyphi B*, and *S. Poona* (cTree) were sorted in to the longer survival nodes for sandy- and clay-loam soils, respectively (**Figure 3.1**). This suggests that the effect of soil-type, and other factors, on survival may be strain-specific; however, investigating potential interactions between strain, soil-type and the other factors included here were outside the scope of the study and should be investigated as part of future research projects.

In the general linear models, strain accounted for up to 18% of variance in survival and 19% of variance in die-off rate, suggesting substantial variation in die-off between strains (**Table 3.2 and 3.3**). Separately from the general linear models' individual log-linear models were developed to compare die-off rates between *Salmonella* strains (**Table 3.4**). By comparing the 95% CI for the die-off rates, we could identify significant differences between strains. For example, *S. Muenchen* had a significantly faster daily die-off rate (-0.060 log CFU or MPN/g per day; 95% CI: -0.055 -0.065), compared to all other strains. Conversely, *S. Braenderup*, *S. Meleagridis*, and *S. Newport-F* had significantly slower daily die-off rates, compared to the other nine strains (**Table 3.4**). *S. Javiana* and *S. Montevideo* had slower die-off rates, than *S. Muenchen*, but had faster die-off rates than *S. Braenderup*, *S. Meleagridis*, and *S. Newport-F* (**Table 3.4**). Interestingly, there was a significant effect of soil-type for 9 of the 12 strains, with daily die-off on average, being slower in sandy-loam soils, compared to clay-loam soils (**Table 3.4**). This supports the conclusion described above that the effect of environmental factors, such as soil-type, on *Salmonella* survival and die-off is strain-specific; however, it may also be a function of the small sample size here.

To our knowledge, our study is one of the first to examine the variation in the survival and die-off of a large number of *Salmonella* strains (n=12) in amended sandy- and clay-loam soils. The use of a multiple strains in studies (not in cocktails) is important, as recent papers (Andino and Hanning, 2015; Harrant et al., 2019) have shown that different *Salmonella* strains can vary substantially in their responses to environmental stresses (e.g., temperature, moisture), which may impact survival. That being said, multiple studies have investigated the survival of a single strain (e.g., You et al., 2006; Semenov et al., 2009; Shah et al., 2019) or a strain cocktail

(e.g., Underthun et al., 2017) in amended and non-amended soils. For example, a study reported the die-off rate for *S. Newport* in soil amended with bovine manure was similar to those reported here (ranged from -0.03 to -0.04 log CFU/g per day (You et al., 2006).

***Salmonella* survival and die-off rates were significantly different in PL amended soils that received weekly irrigation, compared to daily irrigation).**

Three strains (*S. Braenderup*, *S. Newport* F, and *S. Meleagridis*) were exposed to two different irrigation regimes (daily and weekly); all other strains only received daily irrigation. *Salmonella* survived on average 128 d longer (95% CI 101.00, 154.12) when irrigated weekly according to the general linear model (**Table 3.2**). However, *Salmonella* populations were, on average, higher in poultry litter amended soils that received daily irrigation, compared to weekly irrigation (**Figure 3.3, Figure S3.2**). Specifically, the *Salmonella* concentration was approx. 0.7 log<sub>10</sub> lower in pots irrigated weekly as opposed to daily (95% CI -1.0, -0.4;  $P \leq 0.001$ ; **Table 3.3**). According to these models, the *Salmonella* concentration was also approximately 0.4 log<sub>10</sub> higher in poultry litter amended sandy-loam soil, compared to clay-loam soil (95% CI 0.1, 0.8;  $P = 0.008$ ; **Table 3.3**).

Overall, our findings suggest that *Salmonella* die-off was faster under drier conditions (**Table 3.3, Figure 3.3**), which is consistent with past studies that found higher soil moisture content facilitated *Salmonella* survival (Holley et al., 2006; Schwarz et al., 2014; Wang et al., 2018) 1. Similarly, a study that examined *Salmonella* die-off in poultry litter reported that *Salmonella* survived longer when water activity was higher (Himathongkham et al., 1999). While some studies (Danyluk et al., 2007; Underthun et al., 2017) suggest that factors besides soil moisture, such as soil-type and temperature, may have a greater effect on *Salmonella*

survival than soil moisture level, this was not the case in the study reported here. In fact, the magnitude of the effect of irrigation regime on survival and die-off was almost ten-times that of soil type (**Table 3.2** and **3.3**). Irrigation regime (and soil moisture) may also interact with other soil characteristics (e.g., soil particle size, slope) to affect *Salmonella* survival (Sharma and Reynnells, 2018). Although examining the impact of such interactions was outside the scope of the present study, future studies should investigate the effect of such interactions. Given our finding that die-off rates varied significantly between strains, and total survival time appeared to be dependent on both strain and soil-type in the regression tree, future studies should also test to see if the effect of irrigation regime differs substantially between strains.

### ***Salmonella* Survived Significantly Longer in Poultry Litter Amended Soils Compared to Non-Amended Soils.**

Amendment with poultry litter significantly prolonged *Salmonella* Newport E survival by an 89 d (95% CI = 61.32, 116.02,  $P \leq 0.001$ ; **Table 3.2**; **Figure 3.4**; **Figure S3.3**). Overall, these findings are consistent with previous studies (Baloda et al., 2001; Islam et al., 2004a, Holley et al., 2006; You et al., 2006; Nyberg et al., 2010; Ongeng et al., 2011; Hruby et al., 2018; Shah et al., 2019) that reported *Salmonella* survival increased in soils amended with BSAAO (e.g., poultry litter, bovine manures). For example, You and et al. (2006) found that addition of bovine manure to agricultural soils increased *S. Newport* concentrations by as much as 400% within the first three d. Baloda and colleagues (2001) observed *Salmonella* to survive up to 299 d in hog slurry amended agricultural soils, while Hruby and colleagues (2018) detected *Salmonella* in soil samples from poultry litter amended plots nearly a full year after application. Additionally, the die-off rate of *S. Newport-E* was slower in poultry litter amended soils, compared to non-

amended soils (**Table 3.4**). In fact, application of poultry litter amendment increased the population of *Salmonella* by 2.6 (95% CI=2.3, 2.9;  $P<0.001$ ), which was the largest effect observed for any of the factors (e.g., soil-type, irrigation regime, amendment) investigated in the study reported here (**Table 3.3**). The study reported here confirms that application of BSAAO to soils (e.g., poultry litter) facilitates *Salmonella* survival. The 120/90 d application interval outlined by the USDA NOP for growers who use raw manures in produce production is supported by most of the *Salmonella* strains examined in this study (average survival observed was 129 d in amended soils that received daily irrigation). However, there were certain strains that survived >120 dpi; in fact, *S. Braenderup* and *S. Newport-F* survived up to 336 dpi in sandy-loam and clay-loam soil, respectively, under a weekly irrigation regime. This finding suggests that the USDA NOP is sufficient for managing food safety risks associated with BSAAO use under certain conditions, but not all conditions. As such, research into condition-specific modifiers may be needed to better manage these risks due to BSAAO application.

***Salmonella* strain die-off rates were significantly associated with soil-type in PL- and non-amended soils under both irrigation regimes.**

Six general log-linear models were developed to quantify the effect of soil type on *Salmonella* survival (N=3 models) and die-off (N=3 models) in (i) amended pots receiving daily irrigation, (ii) amended pots receiving daily versus weekly irrigation, and (iii) amended versus non-amended pots receiving daily irrigation (**Table 3.2** and **3.3**). According to all three die-off models soil type was significantly associated with *Salmonella* die-off, ( $P<0.001$ ). In fact, the models for amended pots receiving daily irrigation accounted the fixed effects accounted for 17% and 61% of variance in survival and die-off rate, respectively; since the only fixed effects

were trial and soil type, and only soil type was significant we can conclude that the majority of the variance was accounted for by soil type. However, soil type was only associated with survival in the soil type dataset; this may have been a product of the small number of samples available for use in the irrigation (N=18) and amendment (N=6) datasets. Based on the general linear models *Salmonella* die-off was slower in clay-loam soils, compared to sandy-loam soils, which is consistent with past findings. For example, Jechalke et al. (2019) found that *Salmonella* die-off rates were significantly higher in the sand ( $-0.076 \pm 0.014$  log CFU/day), compared to loam ( $-0.034 \pm 0.015$  log CFU/day). Other studies (Platz, 1980; Bech et al., 2010; Fongaro et al., 2017; Underthun et al., 2017) have demonstrated that soil-type impacts *Salmonella* persistence; in addition to other factors, such as temperature and moisture. For example, one study (Platz, 1980) observed higher isolation rates of *Salmonella* in loam- and clay-type soils, compared to sandy type soils. Sandy-type soils typically drain faster with more flow; and thereby, filter microorganisms at a more frequent pace (less-absorptive), compared to loam- and clay-type soils. However, another study (Fongaro et al., 2017) found *Salmonella* survival was significantly lower in clay soil, than in sandy soil. The authors of the study hypothesized clay soils may have more alkaline pH, higher organic matter and higher nutrient contents (due to reduced leaching and better water retention) which may have influenced survival. Thus, the data reported here, and by others, suggests that soil-type can influence *Salmonella* die-off, and in some cases, *Salmonella* survival. These results suggest future studies should investigate additional soil types (i.e., different mixtures of sand, loam and clay) to determine *Salmonella* survival under different field conditions.

## CONCLUSIONS

The study reported here characterized strain variability and the influences of soil-type, irrigation regime, and amendment on the population of *Salmonella*. To our knowledge, this is the first study to quantify the variability for 12 *Salmonella* strains in poultry litter amended soils under different conditions (i.e., soil types) and management practices (i.e., irrigation regimes). In fact, nearly forty years ago, Zibilske and Weaver (1978) described a critique of their *Salmonella* survival in soil study that only a single strain was used, as such the study reported is long overdue. There are some limitations to the study reported here, such as the sample size, and the fact that this was a greenhouse study that cannot precisely replicate field conditions. As such, additional studies with a larger number of samples are needed to determine if these findings are reproducible under field conditions; as well as, for other soil-types (e.g., true sand, loam), irrigation practices (e.g., flood, dry-land production), and soil amendments (e.g., bovine, horse). Despite these limitations, this study is consistent with past research (using single strains, or cocktail), and is one of the first studies to characterize the variability of a diverse number of *Salmonella* strains under various agricultural parameters. Data and findings generated here can be used in (i) future risk assessments, (ii) selection of strains for future challenge and validation studies (e.g., worst case contamination scenarios), and (iii) identification of best practices for application of BSAAO, like poultry litter, to agricultural fields.

Findings from this study suggest a one-size fits all standard for application of BSAAO to agricultural fields would be challenging as there is significant variability in *Salmonella* survival and die-off by strain; as well as variability by environmental and management factors. Moreover, findings suggest obtaining the worse-case contamination scenarios and implement robust recommendations, future studies should critically evaluate strains, soil-type, and management

practices to validate potential interventions and guidance. Based on the results of the study reported here, there are several candidate *Salmonella* strains to use in challenge and validation studies as worst-case contamination scenarios. Prior literature (Harris et al., 2013; Andino and Hanning 2015; Harrand et al., 2019; Ramos et al., 2019) has described the importance of using worst-case contamination scenarios (i.e., conservative approach) and a diverse set of strains, as survival and behavior can vary considerably by factor (e.g., soil-type). For example, *S. Saintpaul* had the longest time to last *Salmonella* detection according to the cTree model (survived 168 d in sandy- and clay-loam amended soils exposed to daily irrigation) (**Figure 3.1**).

Additionally, *S. Braenderup*, *S. Meleagridis*, and *S. Newport-F* each had significantly slower die-off rates, compared to the other nine strains, with *S. Newport-F* being the least of those three influenced by soil-type according to models. Additionally, the use of geographically relevant strains should also be considered to assess persistence and quantify risk of long-term soil contamination, as other studies (Gorski et al., 2011; Strawn et al, 2014; Bell et al., 2015; Jokinen et al., 2015) have demonstrated different *Salmonella* strains can be geographically distributed in specific produce growing regions. For example, *S. Newport-E* (used in the reported study) is geographically relevant to the Eastern Shore of Virginia, which has sandy-loam soil (also used in this study) and has been associated with tomato-borne outbreaks (Greene et al., 2008; Bell et al., 2015). Overall, *Salmonella* survival and die-off is affected by soil characteristics (e.g., soil-type) and management practices (e.g., irrigation regime, amendment), and the effect of these factors may be strain-specific. Thus, data can assist in risk assessment and strain selection for use in challenge and validation studies.

## **ACKNOWLEDGEMENTS**

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**Table 3.1.** Description of the 12 *Salmonella* strains including source, and time to last *Salmonella* detection (survival) in PL amended clay-loam and sandy-loam soils by daily and weekly irrigation

Strain	Source	Time (d) to Last Detect ( <i>Salmonella</i> Survival) <sup>a</sup>					
		Poultry Litter amended				Non-amended	
		Daily Irrigation <sup>b</sup>		Weekly Irrigation <sup>c</sup>		Daily Irrigation	
		Clay-loam	Sandy-loam	Clay-loam	Sandy-loam	Clay-loam	Sandy-loam
<i>S.</i> 4, 12:i:-	Environmental (water)	168	112				
<i>S.</i> Braenderup	Food (cucumber)	210	112	252	336		
<i>S.</i> Enteritidis	Food (romaine lettuce)	168	112				
<i>S.</i> Javiana	Environmental (sediment)	112	210				
<i>S.</i> Meleagridis	Animal (cattle)	84	112	210	252		
<i>S.</i> Montevideo	Food (iceberg lettuce)	210	112				
<i>S.</i> Muenchen	Food (orange juice)	84	84				
<i>S.</i> Newport-F	Food (almond)	168	112	336	252		
<i>S.</i> Newport-E	Environmental (water)	168	112			56	56
<i>S.</i> Paratyphi B	Environmental (sediment)	168	112				
<i>S.</i> Poona	Clinical (human)	168	112				
<i>S.</i> Saintpaul	Clinical (human)	168	168				

<sup>a</sup> Values represent the dpi where at least one of the three replications was positive for enrichment.

<sup>b</sup> Adjusting for the water loss on a daily basis.

<sup>c</sup> Adjusting for the water loss weekly basis.

**Table 3.2:** Results of general mixed models that were developed to investigate the impact of soil type, irrigation regime, and soil amendment on *Salmonella* survival (i.e., total time *Salmonella* was at detectable levels for each pot in this study).

Data Set (R <sup>2</sup> )	Factor	Effect Estimate	95% Confidence Interval	P-Value
Soil Type (0.35) <sup>a</sup>	Trial (One = Reference)			0.188
	Two	-1.17	-19.53, 17.20	0.902
	Three	-15.75	-34.11, 2.61	0.101
	Sandy Soil (Clay = Reference)	-29.56	-44.55, -14.56	< 0.001
	Irrigation (0.73) <sup>b</sup>	Trial (One = Reference)		
Two	-12.83	-45.37, 19.71	0.462	
Three	-17.50	-50.04, 15.04	0.317	
	Sandy Soil (Clay = Reference)	-10.89	-37.46, 15.68	0.444
	Irrigation Regime (Daily = Reference)	127.56	101.00, 154.12	< 0.001
Amendment (0.86) <sup>c</sup>	Trial (One = Reference)			0.109
	Two	14.00	-19.50, 47.50	
	Three	-21.00	-54.50, 12.50	
	Sandy Soil (Clay = Reference)	-23.33	-50.68, 4.02	0.083
	Poultry Litter Amendment (No Amendment = Reference)	88.67	61.32, 116.02	< 0.001

<sup>a</sup> 17% of variance in *Salmonella* survival is explained by the fixed effects shown here, and 18% of variance in *Salmonella* survival is explained by the random effect of strain.

<sup>b</sup> 65% of variance in *Salmonella* survival is explained by the fixed effects shown here, and 8% of variance in *Salmonella* survival is explained by the random effect of strain.

<sup>c</sup> 86% of variance in *Salmonella* survival is explained by the fixed effects shown here since there were no random effects used in the amendment model.

**Table 3.3:** Results of general mixed models that were developed to investigate the impact of soil type, irrigation regime, and soil amendment on *Salmonella* daily die-off rate.

Data Set (R <sup>2</sup> )	Factor	Effect Estimate	95% Confidence Interval	P-Value
Soil Type (0.70) <sup>a</sup>				
	Trial (One = Reference)			0.004
	Two	-0.19	-0.34, -0.04	0.015
	Three	-0.25	-0.40, -0.10	0.001
	Sandy Soil (Clay = Reference)	0.66	-0.54, 0.79	< 0.001
	Daily Die-off Rate	-0.03	-0.03, -0.03	< 0.001
Irrigation (0.71) <sup>b</sup>				
	Trial (One = Reference)			0.034
	Two	-0.24	-0.48, 0.00	0.051
	Three	-0.30	-0.55, -0.06	0.014
	Sandy Soil (Clay = Reference)	0.43	0.24, 0.63	< 0.001
	Irrigation Regime (Daily = Reference)	-0.03	-0.23, 0.18	0.808
	Daily Die-off Rate	-0.02	-0.02, -0.02	< 0.001
Amendment (0.77) <sup>c</sup>				
	Trial (One = Reference)			0.4675
	Two	-0.19	-0.58, 0.21	0.356
	Three	-0.20	-0.60, 0.20	0.331
	Sandy Soil (Clay = Reference)	0.82	0.50, 1.15	< 0.001
	Poultry Litter Amendment (No Amendment = Reference)	2.60	2.25, 2.94	< 0.001
	Daily Die-off Rate	-0.05	-0.05, -0.04	< 0.001

<sup>a</sup> 61% of variance in *Salmonella* survival is explained by the fixed effects shown here, and 9% of variance in *Salmonella* survival is explained by the random effect of strain.

<sup>b</sup> 52% of variance in *Salmonella* survival is explained by the fixed effects shown here, and 19% of variance in *Salmonella* survival is explained by the random effect of strain.

<sup>c</sup> 77% of variance in *Salmonella* survival is explained by the fixed effects shown here since there were no random effects used in the amendment model.

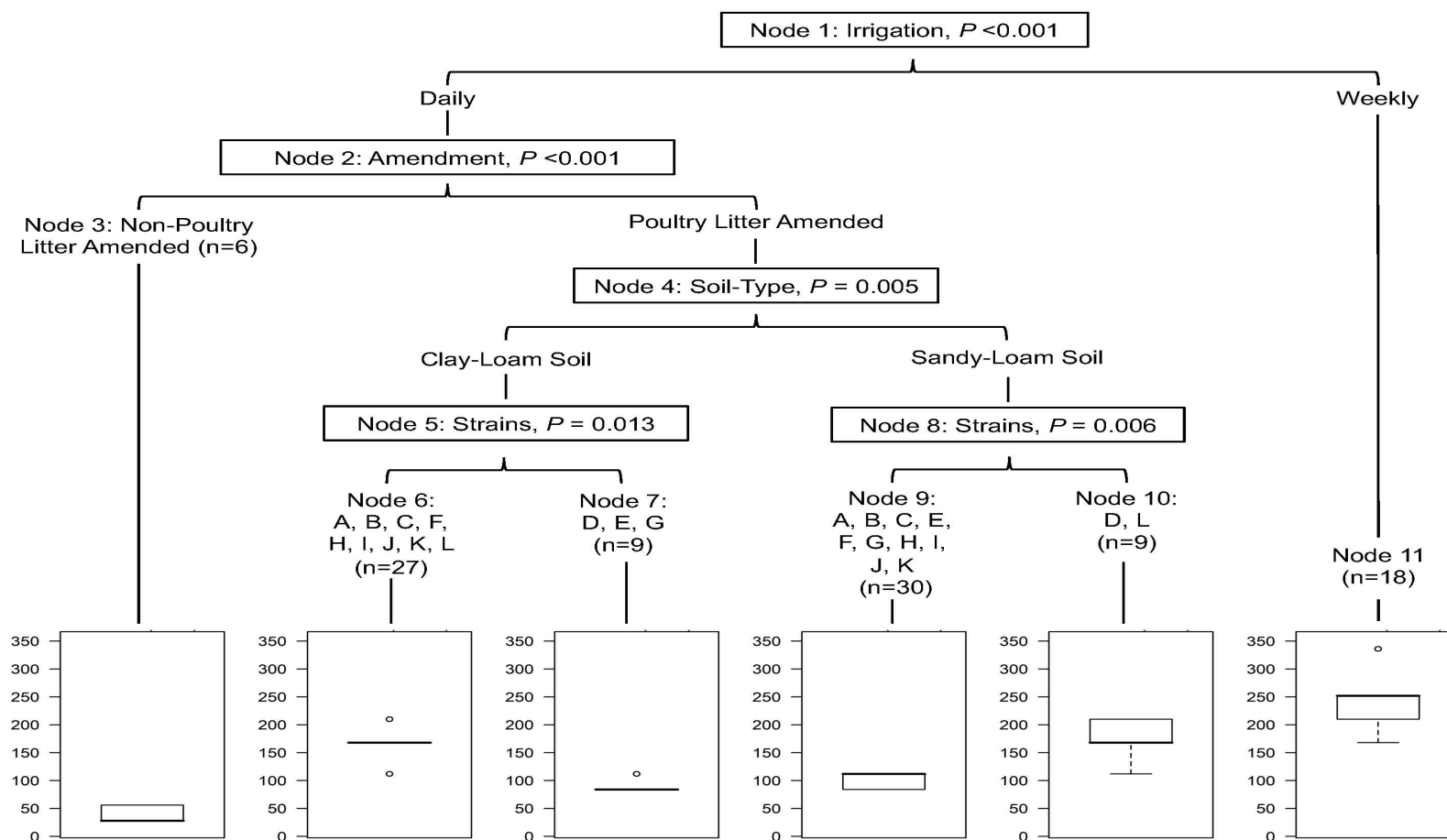
**Table 3.4.** Results of linear models that were developed to compare die-off rate between each of the twelve *Salmonella* strains used herein poultry litter amended soil over time by log linear models

Serovar	Soil-Type (Reference=Clay)			Daily Die-off Rate			Trial 2 (Reference = Trial 1)			Trial 3 (Reference = Trial 2)			R <sup>2</sup>
	Effect <sup>a</sup> Estimate	Standard Error	P-value	Effect <sup>b</sup> Estimate	Standard Error	P-value	Effect <sup>c</sup> Estimate	Standard Error	P- value	Effect <sup>c</sup> Estimate	Standard Error	P- value	
4, 12 I, -	0.32	0.23	0.167	-0.04	0.003	<0.001	-0.04	0.28	0.893	-0.2	0.28	0.463	0.74
Braenderup	0.82	0.15	<0.001	-0.02	0.001	<0.001	-0.22	0.18	0.273	-0.31	0.19	0.097	0.76
Enteritidis	0.68	0.23	0.003	-0.04	0.003	<0.001	-0.05	0.28	0.849	-0.13	0.28	0.653	0.79
Javiana	1.55	0.23	<0.001	-0.03	0.002	<0.001	-0.08	0.27	0.778	-0.19	0.27	0.495	0.83
Meleagridis	0.55	0.16	<0.001	-0.02	0.001	<0.001	-0.19	0.19	0.336	-0.33	0.19	0.091	0.55
Montevideo	-0.08	0.23	0.730	-0.03	0.002	<0.001	-0.31	0.27	0.265	-0.37	0.27	0.178	0.7
Muenchen	1.19	0.24	<0.001	-0.06	0.005	<0.001	-0.27	0.29	0.355	-0.07	0.29	0.809	0.83
Newport F	-0.05	0.15	0.738	-0.02	0.001	<0.001	-0.32	0.19	0.084	-0.3	0.19	0.111	0.63
Newport E	0.70	0.23	0.002	-0.04	0.003	<0.001	-0.21	0.28	0.453	-0.35	0.28	0.220	0.84
Paratyphi	1.47	0.23	<0.001	-0.04	0.003	<0.001	-0.04	0.28	0.877	-0.12	0.28	0.660	0.8
Poona	0.64	0.23	0.006	-0.04	0.003	<0.001	-0.18	0.28	0.519	-0.16	0.28	0.562	0.73
Saintpaul	0.53	0.22	0.017	-0.04	0.002	<0.001	-0.33	0.27	0.232	-0.36	0.27	0.194	0.87

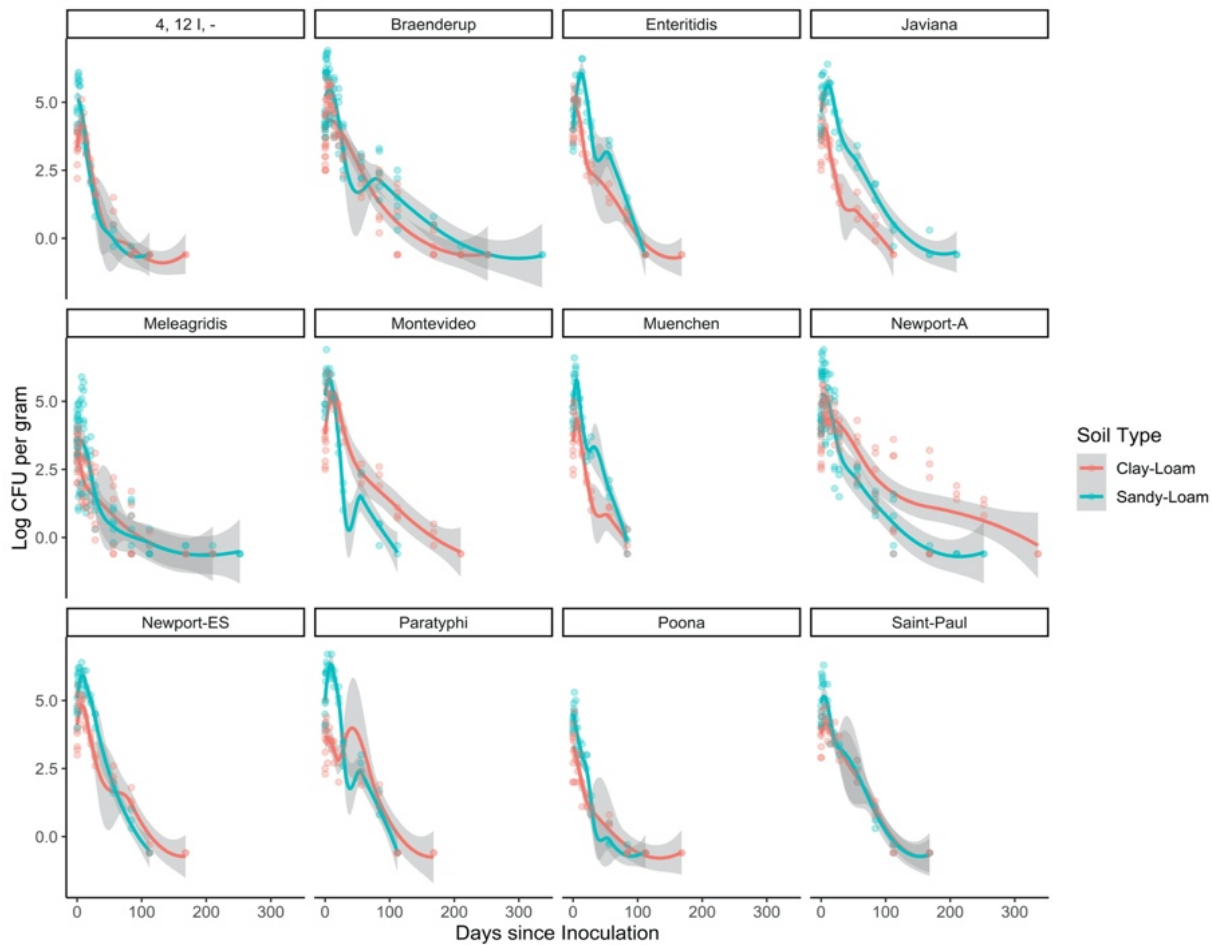
<sup>a</sup> The estimated change in log<sub>10</sub> *Salmonella* population for the given strain when the soil used was sandy loam compared to clay-loam.

<sup>b</sup> The estimated change in log<sub>10</sub> *Salmonella* population for the given strain when for each 1 day increase in time since contamination.

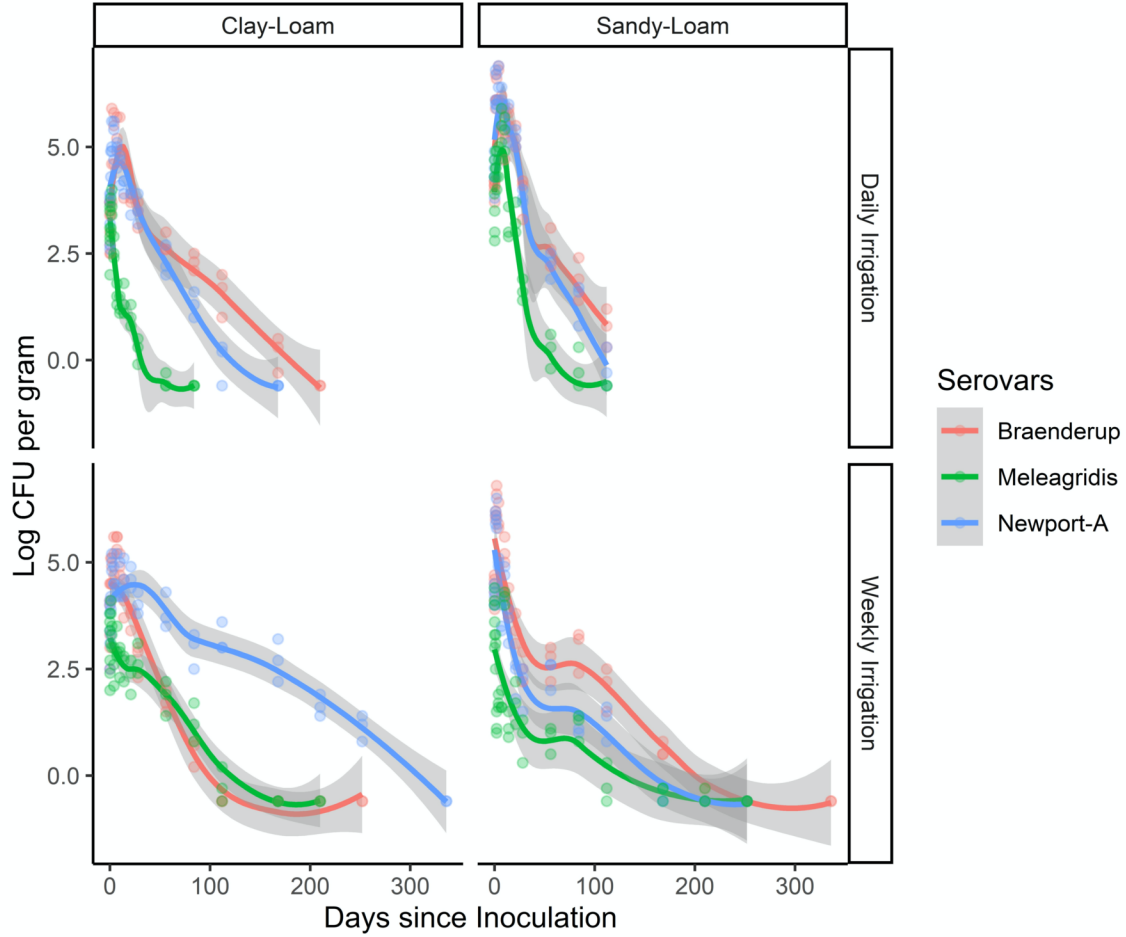
<sup>c</sup> The estimated difference in log<sub>10</sub> *Salmonella* population for the given strain for pots included in trials 2 and 3 compared to trial 1.



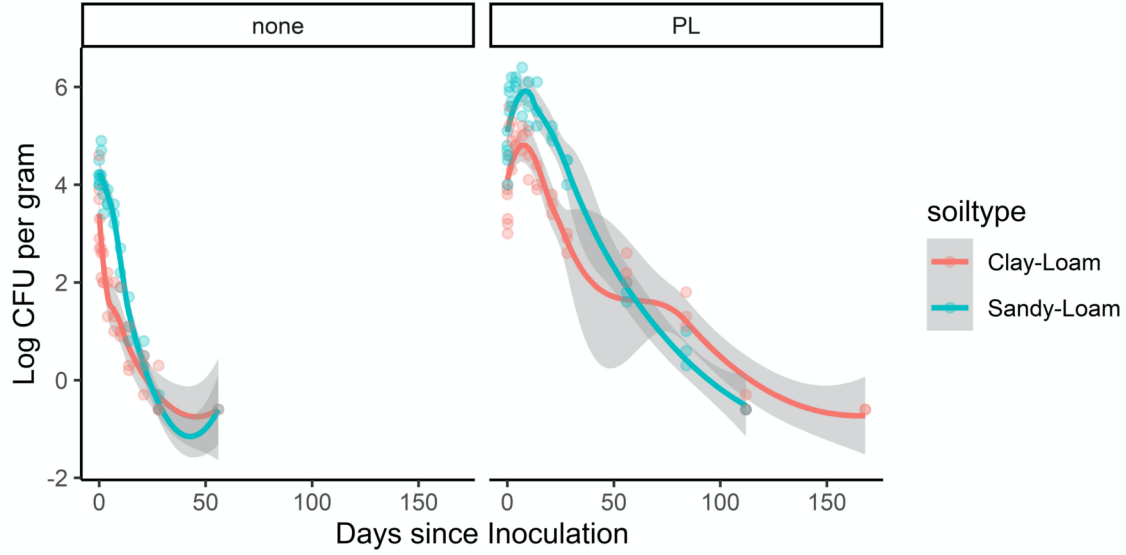
**Figure 3.1.** Conditional inference tree built showing potential interactions in the full dataset between irrigation regime, soil amendment, soil-type and strain, and their impact on the total length of time *Salmonella* remained detectable in the study reported here. For example, *Salmonella* survives the longest in pots receiving weekly irrigation, and the shortest in pots receiving no soil amendment. However, for amended pots receiving daily irrigation, survival time was dependent on soil-type and strain. Bar plots show the expected survival of *Salmonella* (d) in each terminal node. Letters represent the following *Salmonella* strains: A (*S.* 4, 12:i), B (*S.* Braenderup), C (*S.* Enteritidis), D (*S.* Javiana), E (*S.* Meleagridis), F (*S.* Montevideo), G (*S.* Muenchen), H (*S.* Newport-F), I (*S.* Newport-E), J (*S.* Paratyphi B), K (*S.* Poona), and L (*S.* Saintpaul).



**Figure 3.2.** Loess-smoothed regressions showing concentration and 95% confidence intervals (gray shading) for each strain in poultry litter-amended clay-loam (red line) and sandy-loam (teal line) soils.



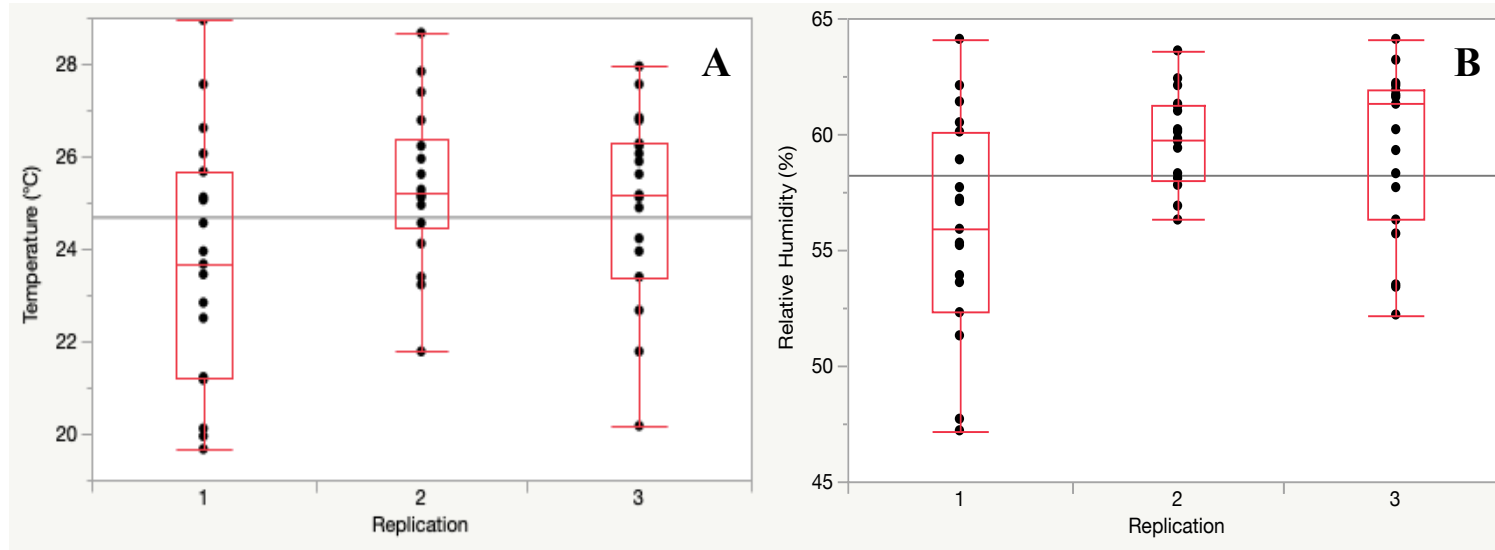
**Figure 3.3.** Loess-smoothed regressions showing concentration and 95% confidence intervals (gray shading) for each strain *S.* Braenderup (red line), *S.* Meleagridis (green line), and *S.* Newport-F (blue line) in poultry litter-amended clay and sandy soils.



**Figure 3.4.** Loess-smoothed regressions showing concentration and 95% confidence intervals (gray shading) for *S. Newport-E* in poultry litter-amended clay-loam (red line) and sandy-loam (teal line) soils.

## Supplemental Materials

**Supplemental Figure S3.1.** Box plots for the temperature (C°; A) and relative humidity (%; B) for each of the three independent trials



**Supplemental Table S3.1.** Physical and Chemical Characteristics of the sadly-loam (SL) and clay-loam (CL) soils<sup>a</sup> used in all experiments

Characteristic	Sandy	Clay
pH	5.7	4.6
Moisture (%)	13	23
Total Organic Carbon (%) <sup>b</sup>	0.5	1.5
Total Nitrogen (mg/kg) <sup>c</sup>	410	1200
Total Kjeldahl Nitrogen (mg/kg) <sup>d</sup>	400	1180
OM (%)	1.0	2.5
Sand (%)	66	41
Silt (%)	24	16
Clay (%)	10	43

<sup>a</sup> SL soil was obtained from a farm on the Eastern Shore of Virginia (Painter, VA, USA), while CL soil was obtained from a farm on mainland Virginia (Petersburg, VA, USA).

<sup>b</sup> Total organic carbon was measured using wet-dry combustion methods and was calculated as loss on ignition (Nelson and Sommers, 2018).

<sup>c</sup> Total nitrogen was measured based on methods described in Mulvaney (Mulvaney, 2018) and Rice et al. (Rice et al., 2017).

<sup>d</sup> Total Kjeldahl nitrogen was determined according to standard method part 4500 N(org) C. Semi-Micro-Kjeldahl methods (Rice et al., 2017).

**Supplemental Table S3.2.** Physical and Chemical Characteristics of the PL<sup>a</sup> amendment used in all experiments

Characteristic	Poultry Litter (PL)
pH	8.1
Moisture (%) <sup>b</sup>	25
Phosphorus (%) <sup>c</sup>	1.8
Total Nitrogen (mg/kg) <sup>d</sup>	3,500
Ammonium nitrogen (NH <sub>4</sub> -N) (mg/kg) <sup>e</sup>	390

<sup>a</sup> Poultry litter (PL) was obtained from a local chicken operation on the Eastern Shore of Virginia (Melfa, VA, USA).

<sup>b</sup> Moisture measured according to Hoskins et al (Peters et al., 2003).

<sup>c</sup> Phosphorous was determined by EPA Method 3015A (US EPA, 2019).

<sup>d</sup> Total nitrogen was determined by combustion (Dumas method) (Rice et al., 2017).

<sup>e</sup> Ammoniacal nitrogen was measured using flow injection analysis of water extracts (Rice et al., 2017).

**Supplemental Table S3.3** Change in concentration (log CFU or MPN/g) between each time point for each *Salmonella* strain in poultry-litter amended sandy-loam soils<sup>a</sup>

Time (Day)	4,12:i-	Branderup	Enteritidis	Javiana	Meleagridis	Montevideo	Muenchen	Newport E	Newport F	Paratyphi	Poona	Saintpaul
0	4.2±0.2 BC <sup>b</sup>	4.1±0.1 DE	4.2±0.2 DE	3.8±0.1 DE	4.3±0.2 BC	4.7±0.2 B	4.7±0.1 CD	4.9±0.1 CD	4.6±0.2 CDE	4.2±0.2 CD	4.0±0.1 ABC	4.5±0.2 CDE
0.17	4.6±0.2 B	4.0±0.2 DE	3.4±0.1 E	4.1±0.2 CDE	3.1±0.2 D	4.7±0.1 B	4.2±0.1 DE	4.4±0.2 D	4.3±0.3 DE	4.7±0.3 C	4.2±0.1 AB	4.2±0.1 CDE
1	5.6±0.2 A	6.2±0.2 AB	4.8±0.2 BCD	5.6±0.2 AB	4.6±0.1 AB	5.9±0.1 AB	5.5±0.3 ABC	5.8±0.2 AB	6.3±0.3 AB	5.9±0.1 AB	4.7±0.1 A	5.5±0.2 AB
2	6.0±0.1 A	6.3±0.2 AB	4.5±0.3 CD	5.6±0.3 AB	4.4±0.3 B	6.4±0.3 A	6.1±0.3 AB	5.8±0.2 AB	6.2±0.3 AB	6.2±0.1 AB	4.8±0.3 A	5.8±0.1 A
4	5.7±0.1 A	6.6±0.3 A	5.5±0.3 ABC	5.5±0.3 AB	4.7±0.2 AB	5.7±0.2 AB	6.2±0.1 A	6.1±0.1 A	6.5±0.2 A	6.4±0.2 A	4.4±0.3 AB	5.8±0.2 A
7	4.5±0.1 BC	5.8±0.2 ABC	5.0±0.1 BCD	5.3±0.2 ABC	5.5±0.2 A	5.8±0.3 AB	5.2±0.1 BC	5.9±0.3 AB	6.1±0.2 AB	6.1±0.1 AB	3.5±0.3 BCD	4.6±0.1 BCD
10	3.7±0.2 CD	5.1±0.2 CD	5.8±0.2 AB	5.8±0.3 A	5.3±0.2 AB	5.5±0.3 AB	5.0±0.1 CD	5.6±0.3 ABC	5.7±0.2 ABC	6.2±0.3 A	3.2±0.2 CD	5.1±0.2 ABC
14	3.3±0.1 D	5.7±0.1 ABC	6.4±0.2 A	5.5±0.2 AB	3.2±0.2 D	5.0±0.1 B	4.2±0.1 DE	5.6±0.3 ABC	5.5±0.3 ABC	5.8±0.2 AB	3.0±0.2 CD	3.9±0.2 DEF
21	2.3±0.2 E	5.2±0.1 BC	4.6±0.2 CD	4.4±0.3 BCD	3.3±0.2 CD	3.5±0.3 C	3.1±0.2 F	5.0±0.1 BCD	5.2±0.1 BCD	5.2±0.2 BC	2.8±0.2 D	3.6±0.1 EF
28	1.2±0.2 F	3.9±0.3 E	4.0±0.2 DE	3.7±0.3 DE	1.6±0.1 E	1.6±0.3 D	3.3±0.2 EF	4.3±0.2 D	3.8±0.1 E	3.2±0.3 DE	1.1±0.2 E	3.2±0.1 FG
56	0.2±0.2 G	2.6±0.3 E	3.3±0.2 E	3.0±0.2 EF	0.2±0.2 F	2.1±0.3 D	1.8±0.2 G	1.8±0.1 E	2.2±0.2 F	2.7±0.2 E	0.0±0.3 F	2.4±0.2 G
84	-0.5±0.1 G	1.9±0.3 F	1.1±0.2 F	1.8±0.2 F	-0.2±0.3 F	0.2±0.3 E	-0.1±0.3 H	0.6±0.2 F	1.4±0.3 F	1.2±0.3 F	-0.5±0.1 F	0.7±0.2 H
112	<-0.6 G(2/3) <sup>c</sup>	0.8±0.3 G	<-0.6 G (2/3)	0.2±0.2 G	<-0.6 F (2/3)	-0.5±0.1 E		<-0.6 G (2/3)	-0.2±0.3 G	<-0.6 G (2/3)	<-0.6 G (2/3)	-0.4±0.1 I
168				-0.3±0.3 G								<-0.6 I (2/3)
210				<-0.6 G (2/3)								

<sup>a</sup> Values presented as log CFU or MPN/g ± standard error; values are averages of a sample from each of the three replications (n=3)

<sup>b</sup> Different letters indicate significant differences ( $P \leq 0.05$ ) by Tukey's honestly significant difference test between rows in a column.

<sup>c</sup> When samples fell below the limit of detection (<-0.6 log MPN/g), samples were enriched for the presence of *Salmonella*, presented in parentheses.

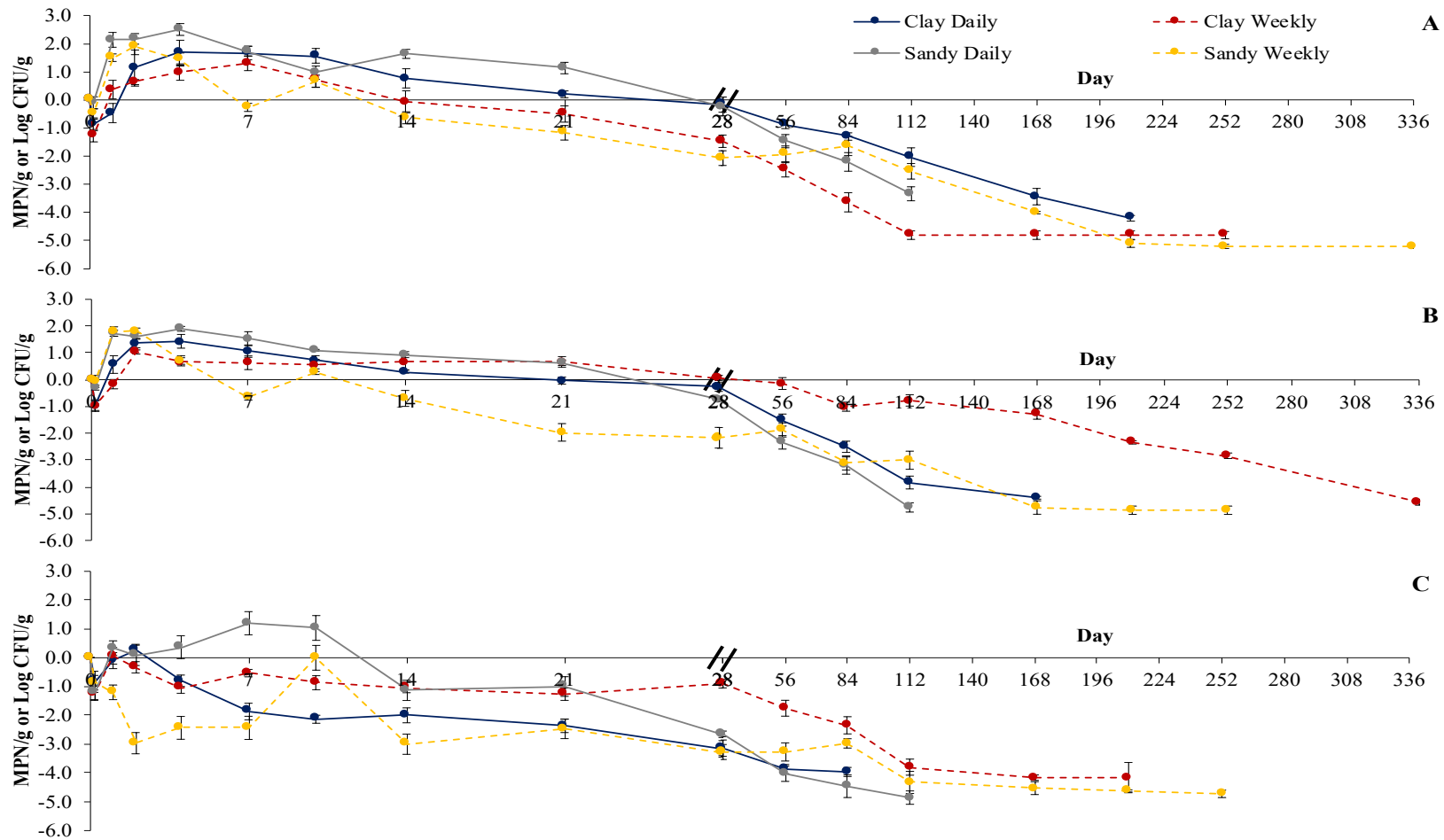
**Supplemental Table S3.4** Change in concentration (log CFU or MPN/g) between each time point for each *Salmonella* strain in poultry-little amended clay-loam soils<sup>a</sup>

Time (Day)	<u>4.12:i-</u>	<u>Branderup</u>	<u>Enteritidis</u>	<u>Javiana</u>	<u>Meleagridis</u>	<u>Montevideo</u>	<u>Muenchen</u>	<u>Newport E</u>	<u>Newport F</u>	<u>Paratyphi</u>	<u>Poona</u>	<u>Saintpaul</u>
0	3.8±0.2 A <sup>b</sup>	3.6±0.1 CDEF	4.0±0.1 B	3.9±0.2 BC	3.4±0.2 AB	3.7±0.1 CD	3.5±0.2 BCD	3.9±0.1 CDE	3.8±0.1 CD	3.8±0.1 AB	3.8±0.2 A	3.8±0.1 AB
0.17	2.7±0.2 BC	2.7±0.1 EFG	3.6±0.1 B	2.7±0.1 DE	2.6±0.3 B	2.6±0.1 E	2.6±0.2 DE	3.2±0.1 EFG	2.8±0.2 DE	2.6±0.2 CDE	2.2±0.2 CDE	3.0±0.1 BC
1	3.5±0.2 AB	3.1±0.3 DEF	5.2±0.2 A	3.6±0.1 CD	3.3±0.3 AB	3.6±0.2 D	4.9±0.1 A	5.1±0.3 A	4.4±0.3 ABC	4.5±0.3 A	3.3±0.1 AB	4.3±0.1 A
2	3.8±0.1 A	4.7±0.6 ABC	5.3±0.1 A	4.8±0.2 AB	3.7±0.2 A	5.7±0.2 A	4.2±0.3 AB	4.8±0.3 AB	5.2±0.2 A	3.9±0.3 AB	4.2±0.2 A	4.2±0.2 A
4	4.0±0.2 A	5.3±0.4 A	5.0±0.1 A	5.0±0.2 A	2.6±0.2 B	5.2±0.3 AB	4.1±0.3 AB	4.9±0.1 AB	5.2±0.3 A	3.3±0.3 BCD	2.6±0.3 BCD	4.3±0.2 A
7	4.5±0.3 A	5.3±0.2 A	5.0±0.1 A	3.8±0.2 C	1.5±0.1 C	5.5±0.2 A	4.2±0.1 AB	5.0±0.1 AB	4.9±0.2 AB	3.7±0.2 ABC	2.7±0.2 BC	4.5±0.2 A
10	4.1±0.3 A	5.2±0.3 AB	4.0±0.1 B	3.4±0.2 CD	1.3±0.1 C	5.1±0.1 AB	3.8±0.2 BC	4.6±0.3 ABC	4.5±0.2 ABC	3.4±0.1 ABCD	2.5±0.3 BCD	3.9±0.2 AB
14	3.5±0.3 AB	4.4±0.3 ABCD	3.5±0.2 B	3.3±0.2 CDE	1.4±0.2 C	4.9±0.1 AB	3.1±0.1 CDE	4.1±0.2 BCD	4.1±0.1 BC	3.1±0.1 BCDE	1.6±0.3 DEF	3.7±0.2 AB
21	2.5±0.2 BC	3.8±0.1 BCDE	2.7±0.2 C	2.3±0.3 EF	1.0±0.1 CD	4.5±0.2 BC	2.1±0.1 EF	3.6±0.1 DEF	3.8±0.2 CD	2.5±0.3 DEF	1.4±0.2 EFG	3.7±0.2 AB
28	1.8±0.2 CD	3.4±0.2 CDEF	2.4±0.2 C	1.6±0.2 FG	0.2±0.2 DE	3.8±0.2 CD	1.4±0.2 FG	2.8±0.1 FG	3.5±0.2 CD	3.3±0.2 BCD	1.0±0.1 FG	3.2±0.3 BC
56	1.0±0.3 D	2.7±0.1 EFG	1.6±0.2 D	1.2±0.3 GH	-0.5±0.1 E	2.3±0.2 E	1.0±0.1 G	2.3±0.2 GH	2.3±0.2 EF	2.2±0.1 EF	0.6±0.1 G	2.4±0.2 C
84	<-0.6 E (3/3)	2.3±0.1 FG	0.8±0.1 E	0.4±0.3 HI	<-0.6 E (3/3)	2.3±0.2 E	-0.2±0.3 H	1.4±0.2 H	1.3±0.2 F	1.5±0.2 F	<-0.6 H (3/3)	1.0±0.1 D
112	<-0.6 E (3/3)	1.6±0.3 GH	<-0.6 F (3/3)	<-0.6 I (2/3)		0.9±0.2 F		-0.5±0.1 I	0.0±0.3 G	<-0.6 G (3/3)	<-0.6 H (3/3)	-0.5±0.1 E
168	<-0.6 E (2/3) <sup>c</sup>	0.2±0.2 HI	<-0.6 F (2/3)			0.1±0.2 FG		<-0.6 I (2/3)	<-0.6 G (3/3)	<-0.6 G (2/3)	<-0.6 H (2/3)	<-0.6 E (2/3)
210		<-0.6 I (2/3)				<-0.6 G (2/3)						

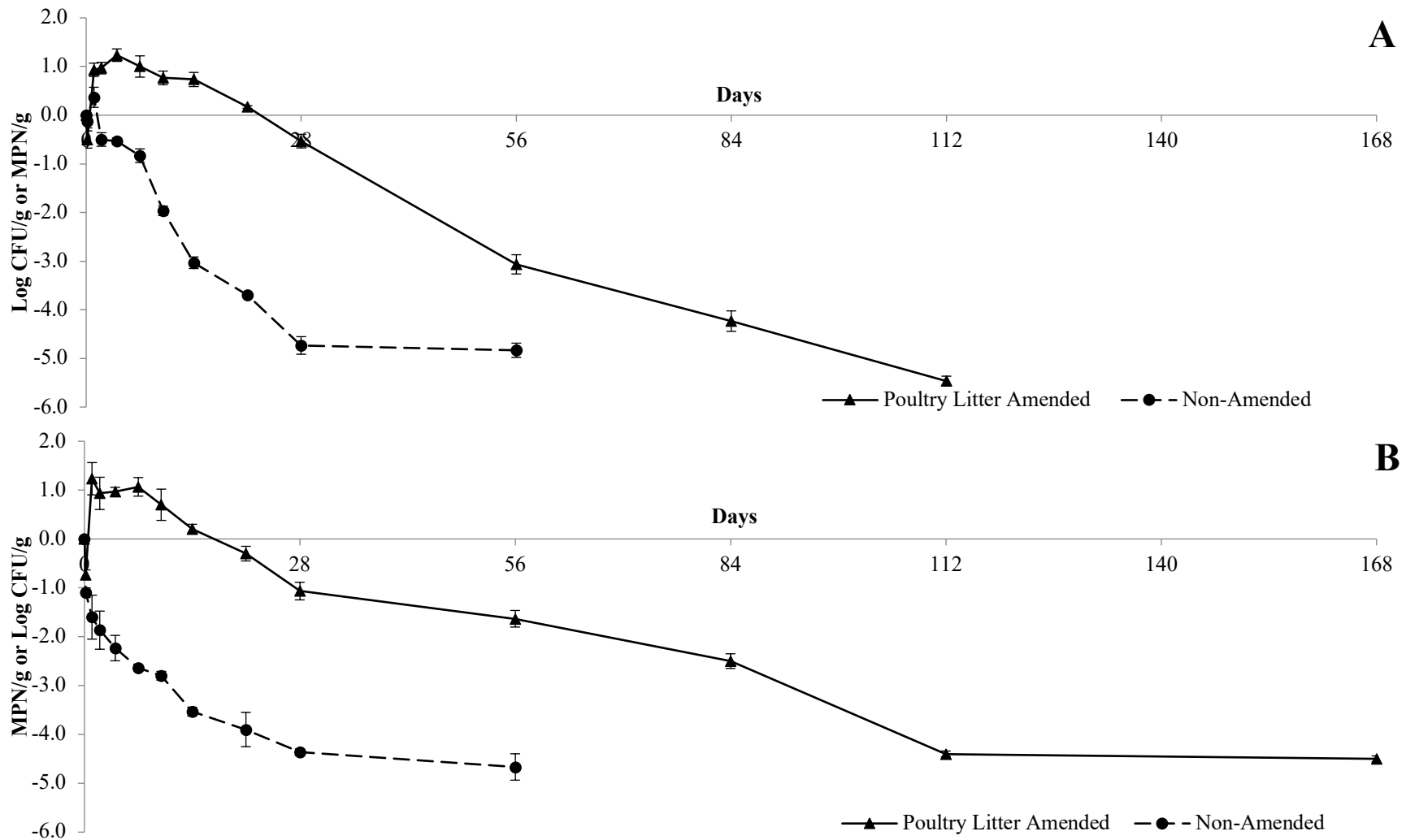
<sup>a</sup> Values presented as log CFU or MPN/g ± standard error; values are averages of a sample from each of the three replications (n=3)

<sup>b</sup> Different letters indicate significant differences ( $P \leq 0.05$ ) by Tukey's honestly significant difference test between rows in a column.

<sup>c</sup> When samples fell below the limit of detection (<-0.6 log MPN/g), samples were enriched for the presence of *Salmonella*, presented in parentheses.



**Supplemental Figure S3.2.** Change in *S. Braenderup* (A), *S. Newport F* (B), and *S. Meleagridis* (C) concentration (log CFU or MPN/g) in poultry litter amended sandy soils irrigated daily (grey solid line) and weekly (yellow dashed line), and in clay soils irrigated daily (blue solid line) and weekly (red dashed line). Error bars represent the standard error of experiments performed in triplicate. The double dashed mark at 28 days represents a change in the x-axis scale from units.



**Supplemental Figure S3.3.** Change in *S. Newport-E* concentration (log CFU or MPN/g) in poultry litter amended (solid line) and non-amended (dashed line) sandy loam (A) and clay loam (B) soils. Error bars represent the standard error of experiments performed in triplicate.

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## Chapter 4: Potential for Internalization of *Salmonella* Newport During Tomato Transplantation

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### Abstract

The risk of *Salmonella* internalization in tomato is of concern due to the challenges in eliminating pathogens during post-harvest activities. The objective of this study was to determine the potential for *Salmonella* to internalize within tomato plants after being exposed to different modes of contamination through the roots after transplanting. Tomato plants were transplanted in triplicate (n=9) and exposed to one of three treatments using a nalidixic acid resistant *Salmonella* Newport strain. The treatments (inoculated at approximately 4 log CFU/g or mL) included contaminated soil (A) and irrigation water administered either once (B, single exposure event) and for 7 d following transplantation (C, repeated exposure event). Plants were sampled 1, 2, 3, 7, and 90 d post-transplantation by surface sanitizing the plant, aseptically sectioning into roots, stems, leaves, flowers, and fruit, and enumerating for *Salmonella* using nonselective and selective media treated with nalidixic acid. Flower and fruit samples were enriched for *Salmonella*. No *Salmonella* was recovered by enumeration and enrichment from flower or fruit samples for all treatments. *Salmonella* was recovered from 29/45 (64%), 0/45 (0%), and 13/45 (29%) of root sections transplanted to treatments A (contaminated soil), B (irrigation water single exposure event), and C (irrigation water repeated exposure event), respectively. Root sections from the contaminated treatment A had significantly higher *Salmonella* recovery, compared to the other treatments ( $P$ -value<0.05) and ranged between  $1.1\pm 0.9$ - $1.9\pm 1.1$  log CFU/root. While *Salmonella* Newport internalized in

tomato root samples following transplantation, *Salmonella* Newport was not observed to internalize in tomato leaf, flower, and fruit samples. Furthermore, data also support a positive association between the increased likelihood of internalization and exposure to contamination.

**Key Words:** Pathogens, Produce, Preharvest, Soil, Water

## **1. Introduction**

Fresh produce can be a source of foodborne illness as it is often consumed raw and generally undergoes minimal processing. According to the Centers for Disease Control and Prevention (CDC) nearly half of the reported foodborne illnesses in the United States are associated with produce (Painter et al., 2013). Between 1998-2008, vine-stalk vegetables, e.g. vegetables that are botanically fruit such as cucumber and tomato, were attributed to 10 and 7% of foodborne hospitalizations and deaths in the US (Painter et al., 2013). *Salmonella enterica* is the leading cause of produce-related bacterial foodborne outbreaks in the US (Hanning et al., 2009) and has been linked to over 100 outbreaks between 1998-2012 involving fresh produce (Wadamori et al., 2017). *Salmonella* Newport is a common serovar implicated in outbreaks associated with fresh produce. For example, between 2002-2010, in the US, there were six multistate outbreaks of *Salmonella* Newport associated with tomato consumption, resulting in 912 cases of salmonellosis (Bell et al., 2015; Bennett et al., 2015; CDC, 2008; Greene et al., 2008). For three of these outbreaks, it was determined that tomato contamination occurred in the pre-harvest environment, such as from contaminated water used for aerial applications (Bennett et al., 2015; CDC, 2008; Greene et al., 2008). Likewise, between 2004-2013 *S.* Newport was linked to 12 seeded vegetable-associated outbreaks, ten of which were

linked to tomatoes (Crim et al., 2018). In fact, water and soil (including the use of biological soil amendments) were determined to be two of the likely sources of produce contamination in the pre-harvest environment (Beuchat, 2006; Hanning et al., 2009; Ilic et al., 2017; Park et al., 2012). Environmental sampling studies (Bell et al., 2015; Strawn et al., 2013a, 2013b) performed in New York State and Virginia (Eastern Shore) also found *Salmonella* in produce field soil and surface water samples.

One possible pre-harvest route of contamination influenced by water and soil is pathogen internalization, the uptake of human enteric pathogens, such as *Salmonella*, through the roots into the plant, and subsequent fruit (Erickson, 2012; Hirneisen et al., 2012). For instance, rootstalk or root hair damage in the plant may present a point of entry for pathogens, as the Casparian strip is not fully formed or is damaged in these circumstance, presents a point of entry to the xylem. Internalization of pathogens into the harvestable portion of the plant (e.g., tomato) is of particular concern because surface sanitation and washing steps are ineffective at removing pathogens once inside plant vasculature tissues. Prior studies (Beuchat et al., 2003; X. Guo et al., 2002; Hintz et al., 2010; Miles et al., 2009; Pollard et al., 2014; Zheng et al., 2013) have investigated pathogen internalization and found varied conclusions, from pathogens being internalized locally in the roots, to pathogens being internalized systematically in the fruits. Two studies (Beuchat et al., 2003; Miles et al., 2009) observed that *Salmonella* was only internalized in root sections (with no further movement within the plant) when tomato plants were irrigated with contaminated water (ca. 5 and 7 log CFU/mL, respectively). However, two other studies (Hintz et al., 2010; Pollard et al., 2014), showed *Salmonella* internalized in stem, leaf, and fruit sections when tomato plants were also irrigated with

contamination water (ca. 7 and 8 log CFU/mL, respectively). Guo et al. (2002) found *Salmonella* internalization occurred in root, stem, and leaf sections when tomato plants were grown in contaminated water in a hydroponic system (ca. 5 log CFU/mL); no tomato fruit was tested for *Salmonella*. Only two study (Beuchat et al., 2003; Zheng et al., 2013), to our knowledge, has investigated *Salmonella* internalization in tomato plants when grown in contaminated soil. Beuchat et al. (2003) observed *Salmonella* internalization in the roots of plants when administered *Salmonella* by direct injection into soil containing plants transplanted 5 days prior to exposure of *Salmonella*. Zheng et al. (2013) observed *Salmonella* internalized in one fruit sample when tomato plants were grown in contaminated soil; soil was contaminated by directly injecting *Salmonella* (ca. 8 log CFU/mL). The majority of studies, except two (Beuchat et al., 2003; X. Guo et al., 2002), observed *Salmonella* internalization in tomato plants using a high starting inoculum level (e.g., 7 or 8 log CFU/mL), which is unlikely to be observed in pre-harvest production environments. Thus, this study aimed to use a lower starting inoculum level to determine if internalization was observed in prior studies because of the large *Salmonella* concentrations. Additionally, no studies directly compare the different contamination routes, such as tomato plants being exposed to contaminated soil and water. Moreover, few studies (Miles et al., 2009) compare the likelihood of *Salmonella* internalization by a single or repeated contamination event.

Therefore, the objective of this study was to investigate the likelihood of *Salmonella* to internalize within tomato plants (specifically the root, stem, leaf, flower, and fruit) after being exposed to three different modes of contamination through the roots following transplanting (using enumeration and enrichment methods). Transplantation

was selected as prior studies (Hallmann et al., 1997; Hirneisen et al., 2012) have identified that internalization is most likely to occur when crops are under stress during transplanting (e.g., establishment of roots, damaged roots). Tomato plants were also selected because of historical *Salmonella* outbreaks associated with the Eastern Shore of Virginia produce growing region. Treatments (A, B, and C) included contaminated soil (A) and irrigation water administered either once (B, single exposure event) and for 7 d following transplantation (C, repeated exposure event). Newport was the serovar of *Salmonella* identified in all six outbreaks linked to the Delmarva.

## **2. Materials and Methods**

### *2.1 Bacterial culture.*

A strain of *Salmonella* Newport (2133) was used in this study. The *S.* Newport strain was adapted to the presence of 50 µg/ml nalidixic acid (N; Sigma Aldrich, St. Louis, MO, USA) through a stepwise exposure process (Parnell et al., 2005).

### *2.2 Inoculum preparation.*

Prior to each replication, *S.* Newport was streaked onto tryptic soy agar treated with 50 µg/ml N (TSAN; Difco, Becton Dickinson, Sparks, MD, USA) and incubated at 35±2°C for 18 h. Following incubation, an inoculum stock was prepared as previously described by Sharma et al. (2016). Briefly, three to five colonies of *S.* Newport were inoculated in 200 ml of tryptic soy broth with 50 µg/ml N (TSBN; Difco, Becton Dickinson) and incubated at 35±2°C for 18 h with agitation (140 rpm). To achieve the target starting inoculum stock, a 10 ml aliquot of the 18 h overnight culture was added to 990 mL of 0.1% peptone (Difco, Becton Dickson) and vigorously shaken. The population of *S.* Newport was determined by plating serial dilutions in 0.1% peptone water on

TSAN. TSAN plates were incubated at 37C for 18 h. The inoculum stock concentration of *S. Newport* was approximately 6 log CFU/ml (which would be diluted further when preparing the treatments).

### *2.3 Inoculation of soil (treatment A) and water (treatment B and C).*

Sandy loam soil, obtained from the Virginia Tech Eastern Shore Agricultural Research and Extension Center (ES AREC), was sifted with a 6.35 mm sieve to remove any large debris or clumps. Contaminated soil (treatment A) was prepared by adding a 100 mL aliquot of the inoculum stock to 1000 g of soil in a sterile Whirl-pak bag (Nasco, Modesto, CA), where it was thoroughly homogenized (combined using a 30 s shake, 30 s rub, 30 s shake procedure, repeated three times). The average *S. Newport* concentration in treatment A (contamination soil) was  $4.3 \pm 0.4$  log CFU/g. Test trial experiments (data not shown) demonstrated uniform inoculation of culture in soil. Plastic pots (6" wide and 7.25" deep; Nursery Supplies Inc., Chambersburg, PA, USA) were filled with 4,000 g of inoculated soil. Contaminated water to be used for irrigation (treatment B and C) was prepared by adding a 10 mL aliquot of the inoculum stock to 990 mL of deionized sterile water in a sterile beaker, where it was vigorously shaken for 30 sec. The average *S. Newport* concentration in treatment B and C (contaminated water to be used for irrigation) was  $4.0 \pm 0.1$  log CFU/ml. Contaminated soil pots were transported to the greenhouse, and contaminated water beakers were stored on ice until irrigation. Controls were also prepared using un-inoculated soil and un-inoculated, sterile, deionized water (treatment D).

### *2.4 Tomato plant preparation and transplantation.*

Tomato plants were grown in triplicate and experiments replicated three times (n=9). Tomato plants (*Solanum lycopersicum*), cultivar Early Girl (Seedway, Hall, NY, USA), were grown from seed in a potting soil medium (Pro-Mix BX; Premier Tech, Québec, Canada) in Styrofoam plugs (Speedling Incorporated, Ruskin, FL, USA) in a greenhouse at the ES AREC under biosafety level 2 containment (Virginia Tech protocol Strawn 15-020). The greenhouse was maintained with an average temperature of  $26\pm 4^{\circ}\text{C}$  and relative humidity of  $65\pm 9\%$  throughout the study according to HOBO Micro Station Data Loggers (Onset Computer Corporation, Bourne, MA). Tomato plants were transplanted between 6-8 wk following seeding to one of the following treatments: A (contaminated soil, irrigated with un-inoculated sterile deionized water), B (un-inoculated soil, irrigated once with contaminated water, single exposure event), C (un-inoculated soil, irrigated with contaminated water for 7 d, repeated exposure event), and D (controls, un-inoculated soil irrigated with un-inoculated, sterile, deionized water). An irrigation event was defined as addition of 250 mL of water each day. Tomato plants were fertilized weekly using Plantex<sup>®</sup> (Master Plant-Prod Inc., Brampton, ON, Canada) until reaching maturity (ca. 90 d).

### *2.5 Sampling, Enumeration, and Enrichment.*

Tomato plants were sampled in triplicate for each of the three replications (n=9). *S.* Newport counts for each tomato plant section were determined at 1, 2, 3, 7 (root, stem, and leaf), and 90 (root, stem in 10 cm portions, leaf, flower, and fruit during the green mature stage) d post-inoculation/transplantation. If counts fell below the limit of detection, tomatoes and flowers were enriched for *Salmonella*. Tomato plants were randomly sampled on d 1, 2, 3, and 7 following transplantation and were aseptically

removed from the soil using sterile gloves, leaving the roots intact. Each tomato plant was submerged in a 70% ethanol (Thermo Fisher Scientific, Waltham, MA, USA) solution and agitated to remove any soil (Hintz et al., 2010; Zheng et al., 2013). The 70% ethanol solution was replaced between each treatment to ensure that no cross-contamination between treatments would occur. Tomato plants were dried in the biological safety cabinet for 10 min or until visibly dry. Once dry, plants were sectioned into root, stem, and leaf samples using a sterile scalpel. Each sample was placed in a Whirl-pak bag (Whirl-pak; Nasco) and 4 ml of 0.1% peptone was added. Each sample (section) was homogenized using a mortar and pestle to expose the vascular tissues. For each sample, serial dilutions were performed and plated in duplicate on TSAN and xylose lysine tergitol 4 agar treated with 50 µg/ml nalidixic acid (XLTN; Difco, Becton Dickinson). Plates were incubated at 35±2°C for 24 h. To obtain the lowest possible limit of detection (<0.6 log CFU/g), 1 ml was plated among three plates (0.3, 0.3, and 0.4 ml/plate) in duplicate. Positive samples were determined by the presence of colonies with the typical *Salmonella* morphology on XLTN (red colonies with black centers indicating H<sub>2</sub>S production).

Once tomato plant fruit reached the green mature stage (approximately 90 d), plants were sampled. The same surface sanitization method described above was used, except the 70% ethanol solution was made in new, un-used large containers (32 gallon plastic garbage bin; ACE, Oak Brook, IL, USA). Tomato plants were individually dried in the biological safety cabinet for 60 min or until visibly dry. Once dry, plants were sectioned into root, stem, leaf, flower and fruit sections using a sterile knife. Stems were aseptically cut into 10 cm (4 in) sections and placed in individual Whirl-Pak bags. Ten

flowers were aseptically picked at random and placed in individual Whirl-Pak bags. Three tomato fruit per plant (n=9 for each treatment) were aseptically picked at random and placed in separate, individual Whirl-Pak bags. Flower and fruit samples were below the limit of detection, so samples were enrichment using a version of the US FDA Bacteriological Analytical Manual (BAM). The remaining flower, and fruit homogenates (sample section and 0.1% peptone mixture) were enriched for *Salmonella* by adding 1.0 and 0.1 mL aliquots of each homogenate to 9 and 9.9 mL of Tetrathionate (TT; Remel, Lenexa, KS, USA) and Rappaport-Vassiliadis (RV; Difco, Becton Dickinson) broths, respectively. TT and RV broths were incubated at 35 and 42±2°C for 24 h, respectively, and then streaked onto XLT and Hektoen enteric agar (HE; Difco, Becton Dickinson). Following a 24 h incubation at 35±2°C, presumptive positive colonies were confirmed by *Salmonella* latex agglutination kit (Microbiology International, Frederick, MD, USA) with C2 group antisera (*S. Newport* belongs to serogroup C2).

### 2.6 Statistical Analysis.

Three tomato plants were sampled in each of the three independent replications (n=9). Each tomato plant was sectioned into the following samples previously described (root, stem, leaf, flower, and fruit). Each treatment group (A, B, and C) contained a total of 45 tomato plants. An analysis of variance was performed using the negative binomial generalized regression model with an elastic net estimation model using JMP Pro 13 statistical software (SAS Institute Inc., Cary, NC.). Comparison of means between treatment, sampling day, and tomato plant section was performed using the Tukey HSD All Pairwise Comparisons test with a significance level of  $P \leq 0.05$ .

## 3. Results and Discussion

This study investigated the likelihood of *S. Newport* to internalize in tomato plants (root, stem, leaf, flower, and fruit) following transplanting when exposure to contaminated soil and water (both from a single and repeated exposure event). The study reported here also aimed to quantify how much *S. Newport* was internalized in tomato plant section samples including the root, stem, leaf, flower, and fruit.

### 3.1 Treatment A (Soil)

#### 3.1.1 Internalization of *S. Newport* in plants (treatment A) 7 d following transplantation.

On average, the concentration of *S. Newport* in the contaminated soil was  $4.3 \pm 0.3$  log CFU/g. Following transplant in contaminated soil (treatment A), *S. Newport* was observed to internalize in 23/36 (64%), 9/36 (25%), and 0/36 of root, stem, and leaf samples within 7 d, respectively (Table 1). Internalization of *S. Newport* was significantly more prevalent ( $P \leq 0.05$ ) in the root section of treatment A compared to treatment B and C (Table 4.1). Average internalization levels were fairly consistent between all sampling days (1, 2, 3, and 7 d post-transplantation) ranging between  $1.1 \pm 0.9$ – $1.9 \pm 1.1$  log CFU/root and  $0.7 \pm 0.2$ – $0.8 \pm 0.6$  log CFU/stem for root and stem sections respectively, all leaf sections were found to be under the limit of detection ( $< 0.6$  log CFU/leaves) for all the sampling days (Table 4.2). Most *S. Newport* internalization occurred within 24 h of transplantation in the contaminated soil, which is similar to results by Zheng and colleagues (2013) who also observed most *Salmonella* internalization within 72 h. Following the study, Zheng et al. (2013) concluded, that newly transplanted plants were more susceptible to internalization of *Salmonella* when exposed within three days following transplantation. Although internalization of *Salmonella* was observed in this study on day 7 following transplantation, it is difficult to

determine whether internalization occurred before the three days following transplantation and persisted in the plant until day 7. More research is needed to determine whether internalization of *Salmonella* is likely to occur through the roots in transplanted tomato plants 3 days following transplantation.

### *3.1.2 Internalization of S. Newport in plants (treatment A) 90 d following transplantation.*

Internalization of *S. Newport* was observed in 6/9 (66.7 %) of the root sections of plants tested 90 d following transplantation (Table 4.33), only 2/9 (22%) of stem sections were found to have *S. Newport* internalization, which occurred only at the base of the stem (0-10 cm) (Table 4.3). The average internalization levels were  $1.7 \pm 0.9$  log CFU/root and  $0.7 \pm 0.2$  log CFU/stem for the root and stem sections respectively. The movement of *S. Newport* in plants was not observed as no other stem portion, leaves, flowers, or fruit were found to have any internalization occur in plants in treatment A (Table 4.3). The Casparian strip in mature roots is a barrier to the entry for *Salmonella*. However, the Casparian strip develops as roots mature so bacterial entry is possible in root tips and branch points where the Casparian strip is incomplete, providing access for the pathogen to move in xylem tissue throughout the plant once internalized. Likewise, Beuchat et al. (2003) found that *Salmonella* internalization was recovered in only the roots (50%) of tomato plants 4 weeks following inoculation but movement was not observed into the stem and leaves of the same plants. Zheng et al. (2013) also observed internalization occur in the root (22%) and stem (11.1%) sections of plants tested, though internalization also occurred in 1 leaf and 1 red ripe fruit sample taken, suggesting the potential of *Salmonella* internal movement from the roots to the fruit of transplanted tomato plants.

This could be due to varying inoculum levels when introducing *Salmonella* to the plants or the amount of transpiration that would help the bacteria move vertically in the plant. Plants in the current study were subjected to soil *Salmonella* levels of  $4.3 \pm 0.3$  log CFU/g which is relatively similar to the *Salmonella* level (4.8 log CFU/ml) Beuchat et al. (2003) administered through injection to plants, while Zheng et al. (2013) subjected plants to a much higher concentration (ca  $10^8$  CFU/ml). Internalization and movement of *Salmonella* in tomato plants through the roots may be dependent on *Salmonella* soil population levels.

### 3.2 Treatment B (Single Exposure Event)

#### 3.2.1 Internalization of *S. Newport* in plants (treatment B) 7 d following transplantation.

On average, the concentration of *S. Newport* in the irrigation water used was  $4.0 \pm 0.1$  log CFU/ml and administered once immediately following transplantation. Internalization of *S. Newport* was not likely to occur after a singular contamination event (Treatment B, contaminated irrigation water administered once). Plants that were irrigated with *Salmonella*-inoculated water only once following transplantation (Treatment B) had little to no plant sections positive for *Salmonella* internalization. Only two stem samples were found to be positive for internalization on days 1, 2, 3, and 7 post-transplantation (Table 4.1). Internalization of *S. Newport* was significantly less prevalent ( $P \leq 0.05$ ) in the root and stem sections of treatment B compared to treatment A and C (Table 1). *S. Newport* population levels were below the limit of detection ( $< 0.6$  log CFU/root and leaf sections) for all sampling days (Table 4.2). For stem sections, only 1 d had levels in the countable range ( $0.6 \pm 0.1$  log CFU/stem), stem sections sampled 2, 3, and 7 d post-transplantation were below the limit of detection (Table 4.2).

### 3.2.2 Internalization of *S. Newport* in plants (treatment B) 90 d following transplanting.

For the plants that were sampled at 90 days following transplanting (Treatment B), no section of the plant (root, stem, leaves, flower, or fruit) was found to be positive for *Salmonella* internalization (Table 4.3). All of these samples were below the limit of detection (Table 4.2). Similar results were found in a study conducted by Miles et al. (2009), where plants exposed to irrigation water contaminated with *Salmonella* Montevideo for one watering period had no *Salmonella* internalization in the fruit, root, stem, and leaf portions of the plants tested. These results suggest that internalization of *Salmonella* in tomato plants from a single incident of exposure through contaminated irrigation water is very unlikely.

### 3.3 Treatment C (Repeated Exposure)

#### 3.3.1 Internalization of *S. Newport* in plants (treatment C) 7 d following transplanting.

The same irrigation water was for this treatment as treatment B, which had a concentration of *S. Newport* in the irrigation water used was  $4.0 \pm 0.1$  log CFU/ml but was administered for seven days following transplanting. Following transplant in soil and irrigating with contaminated water (treatment C), *S. Newport* was observed to internalize in 10/36 (28%), 9/36 (25%), and 0/36 of root, stem, and leaf samples within 7 d, respectively (Table 4.1). Average internalization levels were fairly consistent between all sampling days (1, 2, 3, and 7 d post-transplantation) ranging between  $0.6 \pm 0.1$ – $1.1 \pm 0.7$  log CFU/root and  $0.6 \pm 0.1$ – $0.8 \pm 0.6$  log CFU/stem for root and stem sections respectively, all leaf sections were found to be under the limit of detection ( $<0.6$  log CFU/leaves) for all the sampling days (Table 4.2). These results differ from results found in a study performed by Guo et al. (2002), where root sections of tomato plants were exposed to a

five serotype *Salmonella* cocktail inoculum ( $4.6 \pm 0.1 \log$  CFU/ml) for 9 days in a hydroponic system. Results of this study found that the amount of internalization of *Salmonella* in the roots ( $2.7\text{--}4.4 \log$  CFU/g) and stems ( $<1.5\text{--}3.7 \log$  CFU/g) of the plants varied between the sampling days over the 9-day sampling period, while only one sampling day (day 7) found leaves that were numerated ( $1.1 \log$  CFU/g) while the other sampling days were under the limit of detection which varied between  $<0.98$  and  $1.28 \log$  CFU/g, though 4/5 sampling days had leaves positive for sampling following enrichment. These differences could be due to the fact that plants in the current study were transplanted in uninoculated soil and administered contaminated irrigation water while plants in the study conducted by Guo et al. (2002), were directly exposed to *Salmonella* in the hydroponic solutions, highlighting the fact that soil may act as a filter of the contaminated water lowering the *S. Newport* load that the plant is exposed to at the roots.

### 3.3.2 Internalization of *S. Newport* in plants (treatment C) 90 d following transplanting.

For plants sampled 90 days after transplanting (Treatment C), 2/9 (22%) and 1/9 (11%) of the root and stem portions were found positive for *Salmonella*, respectively (Table 4.3). The average internalization levels were  $1.1 \pm 0.7 \log$  CFU/root and  $0.7 \pm 0.1 \log$  CFU/stem for the root and stem sections, respectively (Table 2). For the stem portion that was found positive, similar to treatment A, internalization was found in the first four-inch section (1-4 inch or 1-10 cm) of the stem near the roots of the plant, all other stem sections were found negative for internalization (Table 4.3). These results suggest that the movement of *S. Newport* through the xylem is difficult once internalized, although studies have found *Salmonella* capable of moving along the vascular tissue in tomato plants. In a study performed by Guo et al. (2001), it was discovered that when the stems

of tomato plants were injected with a cocktail of *Salmonella* before and after flower set, internalization occurred in approximately 40% of the tomatoes tested. Although this is an artificial situation that may not realistically simulate contamination, these suggests that *Salmonella* is capable of moving along the plant's vasculature and into developing fruit on the vine at certain stages of fruit development. Likewise, Gu et al. (2011) found that after injection of *Salmonella typhimurium* into the peduncle of the tomato plant, the bacteria was able to move into the fruit and grow to approximately 6 log CFU/g over a period of two weeks.

Leaves, flowers and fruit were all below the limit of detection and negative following enrichment for flowers and fruit (Table 4.3). Similarly, tomatoes tested in a study conducted by Miles et al. (2009), were negative for *Salmonella* Montevideo internalization even when irrigated with water inoculated at 7 log CFU/ml weekly up to 70 days. Other studies (Hintz et al., 2010; Pollard et al., 2014) have found that *Salmonella* is capable of internalizing into the fruit of tomato plants when exposed at the roots. Hintz et al. (2010), found that 6% of the tomatoes were positive for *Salmonella* internalization, which was confirmed by enrichment and polymerase chain reaction in tomato plants exposed to *Salmonella* Newport through irrigation water at 7 day intervals at 7 log CFU/ml. In a study conducted by Pollard et al. (2014), 1.7 and 3.5% of fruit samples were positive for *Salmonella* internalization from tomato plants inoculated during the flowering stage via root dip with *Salmonella* Newport only and *Salmonella* Newport plus the plant pathogen *Ralstonia solanacearum*, respectively. Further research is needed to determine the role that contamination/inoculum level play in the uptake and transfer of *Salmonella* through the roots and into the fruit of tomato plants. Plants that

experienced internalization of *Salmonella* into the fruit of tomato plants through transfer from the roots were exposed to inoculum levels ranging from 7-8 log CFU/ml; the roots of the tomato plants of the current study were exposed to only 4.0 log CFU/ml. Uptake of *Salmonella* into tomato plants through the roots and movement through the plant and into the fruit may be due to the inoculum level at which the plant is exposed.

#### **4. Conclusion**

Many factors may influence the internalization and movement of *Salmonella* in tomato plants. Studies vary in the type of pathogen exposure, length of pathogen exposure, inoculum level, serotype(s) used, and presence of natural soil microbiota or plant pathogens. Due to these varying factors, it is difficult to compare results and determine which factors most influence the uptake of *Salmonella* through the roots of tomato plants and their subsequent movement through the plant and in some cases into the fruit. Studies that experience internalization of *Salmonella* through the roots and into the fruit vary in the stages of plant growth, root damage, and length and type of *Salmonella* exposure.

Though internalization did not occur in the fruit of the tomato plants transplanted into inoculated soil or irrigated with inoculated water, contamination on the surface of the fruit may occur if it is exposed to contaminated soil or irrigation water. It has been determined that irrigation water that was naturally contaminated with foodborne pathogens could be transferred and contaminate the edible portions of vegetable crops (Mañas et al., 2009; Melloul et al., 2001). Guo et al. (2002b) found that *Salmonella* was capable of surviving for 45 days in moist top soil and that *Salmonella* populations had a 2.5 log CFU/tomato increase on tomatoes that came into contact with the contaminated

soil. To prevent contamination of tomato plants, and more importantly the fruit of tomato plants, it is essential that growers follow good agricultural practices. Although *Salmonella* Newport was found to be capable of internalizing in the roots and some stem portions of tomato plants, it is very unlikely that *Salmonella* Newport is capable of internalizing in the leaf, flower, and fruit when exposed at the roots of tomato plants at a 4 log CFU/g or ml level. More research is needed to determine practices that increase the likelihood of *Salmonella* uptake in tomato plants to develop and implement proper control measures. Internalization events may be associated with other factors such as environmental stressors, serotype, plant growth stage, and inoculation level; thus, further research is needed to determine likely internalization scenarios so as to develop effective control measures.

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## Tables

**Table 4.1** Internalization of *S. Newport* in tomato plant sections 1, 2, 3, and 7 d following transplantation based on treatment type<sup>†</sup>

Day	Roots			Stem			Leaves		
	Treatment <sup>‡</sup>			Treatment			Treatment		
	A	B	C	A	B	C	A	B	C
Day 1	3/9 A <sup>§</sup>	0/9 B	3/9 A	1/9 A	2/9 A	3/9 A	0/9	0/9	0/9
Day 2	8/9 A	0/9 B	3/9 A	4/9 A	0/9 B	4/9 A	0/9	0/9	1/9
Day 3	7/9 A	0/9 B	1/9 B	2/9 A	0/9 A	1/9 A	0/9	0/9	1/9
Day 7	5/9 A	0/9 B	3/9 A	2/9 A	0/9 A	1/9 A	0/9	0/9	0/9
Total	23/36 A	0/36 C	10/36 B	9/36 A	2/36 B	9/36 A	0/36	0/36	2/36

<sup>†</sup> Values based on the presence/absence of *Salmonella* Newport with a <0.6 log CFU/g limit of detection.

<sup>‡</sup> Separated by the treatment of the introduction of *Salmonella* by inoculated soil (A), irrigation water for 1 day (B), and irrigation water for 7 days (C).

<sup>§</sup> Letters represent significance between the different treatments (A, B, and C) of the roots and stem ( $P \leq 0.05$ ).

**Table 4.2** Internalization levels (log CFU/root, stem or leaves  $\pm$  standard deviation) of *S. Newport* in tomato plant sections 1, 2, 3, 7 and 90 d following transplantation<sup>†</sup>

Day	Treatment A <sup>‡</sup>			Treatment B			Treatment C		
	Root	Stem	Leaves	Root	Stem	Leaves	Root	Stem	Leaves
Day 1	1.1 $\pm$ 0.9	0.7 $\pm$ 0.2	<0.6	<0.6	0.6 $\pm$ 0.1	<0.6	1.0 $\pm$ 0.8	0.7 $\pm$ 0.2	<0.6
Day 2	1.7 $\pm$ 0.9	0.8 $\pm$ 0.6	<0.6	<0.6	<0.6	<0.6	1.0 $\pm$ 0.8	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1
Day 3	1.9 $\pm$ 1.1	0.8 $\pm$ 0.6	<0.6	<0.6	<0.6	<0.6	0.6 $\pm$ 0.1	0.7 $\pm$ 0.2	0.7 $\pm$ 0.1
Day 7	1.4 $\pm$ 1.1	0.7 $\pm$ 0.2	<0.6	<0.6	<0.6	<0.6	0.8 $\pm$ 0.4	0.8 $\pm$ 0.6	<0.6
Day 90	1.7 $\pm$ 0.9	0.7 $\pm$ 0.2	<0.6	<0.6	<0.6	<0.6	1.1 $\pm$ 0.7	0.7 $\pm$ 0.1	<0.6

<sup>†</sup> Values are the average of triplicate samples from three replications (n=9).

<sup>‡</sup> Separated by the treatment of the introduction of *Salmonella* by inoculated soil (A), irrigation water for 1 day (B), and irrigation water for 7 days (C).

**Table 4.3** Internalization of *S. Newport* in tomato plant sections 90 d following transplantation<sup>†</sup>

Treatment <sup>‡</sup>	Roots	Stem <sup>§</sup>								Leaves	Flowers	Fruit
		0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80			
A	6/9	2/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
B	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C	3/9	1/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9

<sup>†</sup> Values based on the of presence/absence of *Salmonella Newport* with a <0.6 log CFU/g limit of detection.

<sup>‡</sup> Separated by the treatment of the introduction of *Salmonella* by inoculated soil (A), irrigation water for 1 day (B), and irrigation water for 7 days (C).

<sup>§</sup> Stems were divided into 10 cm (4 in) sections starting at the base after the roots.

**Chapter 5: Influence of soil population and root damage on the internalization of  
*Salmonella* Newport in transplanted bell pepper plants**

*Formatted for the journal of Food Microbiology*

**Abstract**

The objectives of this study were to investigate *Salmonella* internalization in transplanted pepper plants exposed to different levels of soil contamination and determine the role root damage plays in *Salmonella* Newport uptake. Plant's roots were left intact, damaged, or removed and placed in inoculated water (6.2 log CFU/ml). Pepper plants in the soil contamination study, were transplanted into soil inoculated with *Salmonella* at different contamination levels. Samples were collected 1, 2, 3, and 7d post-transplant. Plants were surface sanitized and enumerated for *Salmonella* internalization by plant section.

Internalization of *Salmonella* in the stems (no roots: 6.8±0.1, damaged roots: 3.0±0.5, and intact roots: 1.9±0.3 log CFU/stem) and leaves (no roots: 1.2±0.1, damaged roots: 1.4±0.2, and intact roots: 3.6±0.6 log CFU/stem) of plants significantly differed based on root treatment. In plants exposed to high contamination *Salmonella* internalized in 97.2% (2.2 log CFU/root) and 16.7% (0.8 log CFU/stem) of root and stem sections respectively. In plants exposed to medium contamination, *Salmonella* internalized in 50% (0.8 log CFU/root) of root sections of the plants. In low *Salmonella* contaminated soils, no internalization occurred in root and stem samples. Due to the results internalization was highly dependent on root damage and the level of soil contamination.

## 1. Introduction

Fresh fruits and vegetables have the potential to be contaminated with foodborne pathogens at various stages of the production process. At the preharvest level, contamination of fresh produce may occur through the soil, irrigation water, animal inclusion, or worker hygiene. It was determined that preventative actions to target reduced soil and irrigation water were the most effective way to control the produce contamination at preharvest (Ilic et al., 2017; Park et al., 2012; Powell et al., 2002). *Salmonella* is a leading cause of produce related bacterial foodborne outbreaks (Painter et al., 2013). Of the *Salmonella* serovars commonly associated with foodborne outbreaks, *Salmonella* Newport is commonly associated with fresh produce outbreaks. Between 2004-2013 *S. Newport* was attributed to 22 fresh produce outbreaks in the U.S., 12 of those outbreaks were linked to seeded vegetables (i.e. tomatoes) (Crim et al., 2018). Between 2002-2012, *Salmonella* was isolated from 21 different pepper samples taken by the U.S. Department of Agriculture Microbiological Data Program, with Newport being one of the more prevalent serovar isolated (Reddy et al., 2016)

Along with surface contamination, internalization of foodborne pathogens is a concern, making surface sanitizers or treatments ineffective in reducing the presence of foodborne pathogens. Pathogens such as *Salmonella* and *E. coli* O157:H7 have been found capable of acting as endophytes, becoming internalized in the tissue of a plant without causing any harm or distress (Fan et al., 2009). Endophytic bacteria are capable of entering the plant through root hair cells and are then transported through the vascular tissue (Hallmann et al., 1997; Lamb et al., 1996; Sturz et al., 1999). Endophytic bacteria commonly exist in the soil, manure, and irrigation water and may be introduced to plants

during transplanting (Beuchat, 2006; Brandl, 2006). It has been suggested that root damage may influence the internalization of *Salmonella* and its movement throughout the rest of the plant (Deering et al., 2012). While a study conducted by Guo et al. (Guo et al., 2002) found that cut roots did not influence the internalization of *Salmonella* in tomato plants grown hydroponically in an inoculated nutrient solution. Colonization and migration of *Salmonella* has also been attributed to rot epidermal breakage (Cooley et al., 2003). Other studies suggest that lateral roots present sites of entry and colonization for *Salmonella* (Karmakar et al., 2018; Klerks et al., 2007). Little is understood on how damage to the lateral roots and exposure to the vascular tissue influences the internalization and movement of *Salmonella* from the roots to the stem and leaves of the plant.

Many studies have investigated the potential for *Salmonella* to transfer from the roots to other sections of plants, but results vary drastically. One potential factor that may influence varying results is the inoculation level to which plants are exposed. In tomato plants exposed to *Salmonella* at the roots at high concentrations (7-8 log CFU/ml) following transplanting or early in plant development, internalization occurred in the roots, stem, leaves, and in some cases the fruit (Hintz et al., 2010; Pollard et al., 2014; Zheng et al., 2013). Alternatively, in tomato plants exposed to lower *Salmonella* concentrations (4-5 log CFU/ml), internalization only occurred in the roots and stem of the plants, and little to no internalization occurred in the leaves and the fruit of the plants (Beuchat et al., 2003; Liu et al., 2018). Likewise, previous results in Chapter 3 demonstrated similar results in plants exposed to soil and water at 4 log CFU/g and CFU/ml, with internalization occurring in the roots and stem of tomato plants and no

internalization occurring in the fruit. Though *Salmonella* has been isolated naturally in agricultural environments (Bell et al., 2015; Strawn et al., 2013b, 2013a; Truitt et al., 2018), it is unlikely that *Salmonella* populations reach large concentrations in the environment. These results call into question the role *Salmonella* concentration exposure to plant roots plays into the internalization and transfer to other areas of the plant. The objectives of this study were to; (i) determine how root damage and the root stalk influence the internalization and movement of *Salmonella* Newport in bell pepper plants, and (ii) investigate how varying *Salmonella* soil populations influence internalization in transplanted bell pepper plants.

## **2. Materials and Methods**

### *2.1 Bacterial culture*

An environmental strain of *Salmonella* Newport (2133) was used for this study. The *S.* Newport strain was conditioned to grow in the presence of 50 µg/ml nalidixic acid (N; Alfa Aesar, Haverhill, MA, USA) through a gradual exposure process (Parnell et al., 2005).

### *2.2 Pepper plant preparation*

Pepper plants were grown in triplicate and the experiments were performed three times for both studies described (n=9). Bell pepper plants (*Capsicum annuum*), cultivar Hybrid New Ace (Seedway, Hall, NY, USA) were grown from seed in a potting soil medium (Pro-Mix BX; Premier Tech, Québec, Canada) in Styrofoam plugs (Speedling Incorporated, Ruskin, FL, USA) in a growth chamber (Percival, Perry, Iowa) maintained at 26 °C and 60% relative humidity. Plants were irrigated daily with sterile water and fertilized twice a week using Plantex<sup>®</sup> (Master Plant-Prod Inc., Brampton, ON, Canada)

at manufacturers recommended concentration levels. Plants were used for their respective experiments 5 weeks following seeding.

### *2.3 Water inoculation for root damage study*

Before each replication, *S. Newport* was streaked onto xylose lysine deoxycholate agar treated with 50 µg/ml nalidixic acid (XLD-N; Difco, Becton Dickinson, Sparks, MD, USA) and incubated for 24 h at 35 °C. Following incubation, one colony was transferred into 10 ml of tryptic soy broth treated with 50 µg/ml nalidixic acid (TSB-N; Difco, BD) and incubated for 24 h at 35 °C. From the overnight culture a 10 µl loop was transferred to 10 ml of TSB-N and incubated for 24 h at 35 °C. Following incubation, the culture was washed twice by centrifuging at 3,000 x g for 5 min (Allegra X-14, Beckman Coulter, Fullerton, CA), discarding the supernatant, and suspending in 5 ml of 0.1% peptone (Fisher Bioreagents, Thermo Fisher Scientific, Waltham, MA). The inoculum stock culture population level of *S. Newport* was  $\sim 10^8$  CFU/ml. The washed *S. Newport* culture diluted by adding 1 ml into 9 ml 0.1% peptone and then adding 1 ml of the diluted peptone into 9 ml of sterile deionized water and agitated to distribute evenly throughout the water. The average *S. Newport* concentration in the water was  $6.2 \pm 0.3$  log CFU/ml.

### *2.4 Root Treatments*

Pepper plants were carefully removed from the plug, in order to avoid potential root damage, and roots were rinsed with sterile deionized water to remove majority of the potting soil. Following rinsing, plants were randomly assigned to one of three treatments: intact roots, damaged roots, and no root stalk. The roots of the plants in the intact root treatment were left unaltered following rinsing. Lateral roots from the plants in the damaged root treatment were cut with a sterile blade removing roughly half of the lateral

root length. In the no root stalk treatment, the root stalk of plants was removed using a sterile blade under sterile deionized water to ensure that air did not enter the vascular tissue of the plant. Following treatments plants were placed into a 15 ml Falcon® tube (Corning Inc., Corning, NY) containing the 10 ml aliquots of *S. Newport* inoculated water so that only the roots (or stem for plants with the root stalk removed) were in contact with the inoculated water. Plants were left in the inoculated water under a biological safety cabinet for 24 h.

### *2.5 Sampling and enumeration of S. Newport internalization in root treatment plants*

Plants were removed from the *S. Newport* inoculated water and surface sanitized by submerging and agitating plants in 70% ethanol (Fisher Scientific) for 30 sec. Plants were placed under the biological safety cabinet to allow residual ethanol to evaporate and left until plants were visibly dry. Once dry, plants were sectioned into root, stem and leaf samples using a sterile blade. Each sample was placed in a Whirl-pak bag (Whirl-pak; Nasco) and 10 ml of 0.1% peptone was added. In order to expose the vascular tissue, samples were macerated using a mortar on the bench top. Serial dilutions were performed for each sample and plated in duplicate on tryptic soy agar treated with 50 µg/ml Naladixic Acid (TSA-N; Difco, BD) and XLD-N. In order to obtain the lowest limit of detection where needed (<1 log CFU/plant section), 1 ml of the sample was plated across four plates (250 µl per plate) in duplicate. Plates were incubated at 35°C for 24h. Following incubation, plates were enumerated.

### *2.6 Inoculation of soil and transplanting of pepper plants for soil population study*

Before each replication, *S. Newport* was streaked onto XLD-N and incubated for 24 h at 35 °C. Following incubation, 3 colonies were transferred into 50 ml of TSB-N

and incubated for 24 h at 35 °C. Following incubation, the culture was washed by centrifuging at 4,000 X g for 15 min, discarding the supernatant, and suspending in 25 ml of 0.1% peptone. The inoculum stock culture population level of *S. Newport* was  $\sim 10^9$  CFU/ml. The stock culture was diluted to obtain two additional target inoculum population levels ( $\sim 10^7$  and  $\sim 10^5$  CFU/ml) by adding 500  $\mu$ l and 5  $\mu$ l of the original stock culture to 50 ml of 0.1% peptone, respectively. Previously sifted and dried sandy loam soil, obtained from the Virginia Tech Eastern Shore Agricultural Research and Extension Center (Painter VA), was inoculated by adding 50 ml of one of the three inoculums with varying *S. Newport* population levels ( $\sim 10^9$ ,  $\sim 10^7$ , and  $\sim 10^5$  CFU/ml) into 1000 g of soil in a gallon Ziploc® bag (SC Johnson, Racine, WI) where it was thoroughly homogenized through a procedure of shaking for 30 sec, massaging for 30 sec, and shaking for 30 sec repeated three times. The average *S. Newport* concentration in the soil for each treatment was  $6.6 \pm 0.8$  CFU/g (high),  $4.0 \pm 0.7$  CFU/g (mid), and  $2.8 \pm 0.2$  (low) log CFU/g. Following inoculation, 500 g of the inoculated soil was then transferred into a 4" x 4" x 4" pot (Nursery Supplies Inc., Chambersburg, PA, USA). A pepper plant was transplanted from the plug into one of the three soil population levels. Controls were also prepared using un-inoculated soil. Plants were stored and sampled along a 7-day period in a growth chamber maintained at  $25 \pm 2$  °C and  $55 \pm 10\%$  relative humidity.

### *2.7 Sampling, enumeration, and identification of S. Newport in soil population level plants*

Plant soils were sampled 1, 2, 3, and 7 days following transplanting. Soil was sampled by weighing out a 25 g sample from each of the three treatments (high, mid, and low) into a Whirl-pak bag and diluting with 0.1% peptone. Serial dilutions were

performed, and samples were plated on XLD-N and TSA-N then incubated at 35 °C for 24h. Plants were sampled and sectioned as previously mentioned. Diluting, plating, and enriching was conducted following a modified method performed by Guo et al. (2002). Briefly, 5 ml of 0.1% peptone was added to each sample and the sample was homogenized as described previously. Samples were plated in duplicate on TSA-N and XLD-N and incubated at 35 °C for 24 h. In order to obtain the lowest limit of detection (<0.7 log CFU/section), 1 ml of the sample was plated across four plates (250 µl per plate) in duplicate. Following incubation, plates were enumerated for *Salmonella*. Following the first replication, due to background interference, samples were no longer plated on TSA-N for the final two replications. The remaining peptone and plant tissue were enriched by adding 5 ml Universal Preenrichment Broth (UPB; Difco, BD) and incubated at 35 °C for 24h. The remaining enrichment process was performed following a version of the US FDA Bacteriological Analytical Manual (BAM) (FDA, 2019). The samples were enriched for *Salmonella* by adding 1.0 and 0.1 ml aliquots of homogenates below the limit of detection to 9 and 9.9 ml of Tetrathionate (TT; Remel, Lenexa, KS) and Rappaport-Vassiliadis (RV; Difco, BD) broths, respectively. TT and RV broths were incubated at 35 and 42 °C for 24 and 48 h, respectively. Following incubation, enrichment broths were streaked onto XLD-N and Hektoen Enteric agar treated with 50 µg/ml Naladixic Acid (HE-R; Difco, BD) and incubated at 35 °C for 24 h. Colonies were randomly selected to confirm presumptive *Salmonella* colonies from TSA-N, XLD-N, and HE-R plates by *invA* gene (a 250 bp amplicon) PCR amplification by following previously described methods (McEgan et al., 2014), using *invA* primers

GAATCCTCAGTTTTTCAACGTTTC and TAGCCGTAACAACCAATACAAATG  
(Integrated DNA Technologies, Coralville, IA).

### 2.8 Statistical Analysis

Statistical analysis was performed using JMP Pro 15 statistical software (SAS Institute Inc., Cary, NC). Comparison of means was conducted to determine significant difference between sampling day, treatment, and plant section using the Tukey HSD All Pairwise Comparison test. Significant differences between presence/absence samples were determined by performing an analysis of variance using a binomial generalized regression model with a chi-square effects test. Significance was determined with a P-value  $\leq 0.05$ .

## 3. Results

### 3.1 Influence of root damage on the internalization and movement of *Salmonella* Newport in pepper plants.

A total of 27 plants were sampled during this experiment (9 intact roots, 9 damaged roots, 9 no root stalk) over the course of three replications (n=9). Plants were left in water inoculated with *S. Newport* at  $6.2 \pm 0.3$  log CFU/ml for 24 h. Population counts for *Salmonella* were significantly ( $P < 0.05$ ) lower on XLD-N (data not shown) compared to TSA-N plates.

There was no significant ( $P > 0.05$ ) difference in the levels of *S. Newport* internalization in the roots of plants with intact roots and damaged roots ( $5.3 \pm 0.2$  and  $5.5 \pm 0.2$  log CFU/roots, respectively) regardless of treatment (Fig. 5.1). The transfer of the *S. Newport* from the roots to the stem was significantly ( $P < 0.05$ ) different between the intact and damaged roots. The uptake of *S. Newport* in the stem of plants in the no

root treatment was significantly higher ( $P < 0.05$ ) than the stems of plants in the damaged root and intact root treatments, with levels varying between  $6.9 \pm 0.1$ ,  $3.0 \pm 0.5$  and  $1.9 \pm 0.3$  log CFU/stem, respectively (Fig. 5.1). Plants with the root stalks removed (no root) had significantly ( $P < 0.05$ ) higher internalization of *S. Newport* occur in the leaves,  $3.6 \pm 0.6$  log CFU/leaves, than the plants in the damaged and intact root treatments ( $1.4 \pm 0.1$  and  $1.2 \pm 0.1$  log CFU/leaves respectively) (Fig. 1).

### *3.2 Influence of Salmonella Newport soil population on the internalization in pepper plants.*

A total of 120 plants were sampled during this experiment (12 control, 36 high, 36 mid, and 36 low treatment plants) over the course of three replications. A soil sample was collected for each treatment (high, mid, and low) on each sampling day (1, 2, 3, and 7 d post-transplanting) for each replication ( $n=3$ ). No control plant or soil samples were positive for *Salmonella* across all sampling days and replications. The starting *S. Newport* soil population was  $6.6 \pm 0.8$ ,  $4.0 \pm 0.7$ , and  $2.8 \pm 0.2$  log CFU/g for the high, mid and low treatments respectively (Figure 5.2). The *S. Newport* populations were not significantly ( $P > 0.05$ ) different between 0-3 d for all three treatments. By day 7, *S. Newport* population levels were significantly ( $P < 0.05$ ) lower for the high ( $4.8 \pm 0.3$  log CFU/g) and low (1.4 log CFU/g) treatments, no significant population differences ( $P > 0.05$ ) were observed between sampling days for the mid treatment. Treatments were significantly ( $P < 0.05$ ) different for each sampling day except for day 7 where there was no significant difference ( $P > 0.05$ ) between high and mid treatments and between mid and low treatments.

#### *3.2.1 Internalization levels of S. Newport in plants in high, mid and low treatments*

Following transplanting, *S. Newport* was observed to internalize in the root sections of plants in the high and mid treatments across all sampling days. Root sections of plants in the low treatment were below the limit of detection ( $<0.7$  log CFU/root) (Table 5.1). Root internalization levels of *S. Newport* for high and mid treatments were fairly consistent between sampling days and ranged from  $1.8\pm 0.2$  to  $2.4\pm 0.3$  and  $0.7\pm 0.1$  to  $1.2\pm 0.2$  log CFU/root respectively. Root sections of plants transplanted into the high treatment had significantly ( $P < 0.05$ ) higher levels of *S. Newport* internalization compared to plants transplanted into the mid and low treatments across each sampling day. No *S. Newport* internalization was detected ( $<0.7$  log CFU/stem) in the stem sections of the plants in the mid and low treatments. Internalization levels were consistently low ranging between  $0.8\pm 0.1$  and  $0.9\pm 0.2$  log CFU/stem between day 1-3 for stem sections of plants in the high treatment, while all stem samples of plants on day 7 were below the limit of detection. All leaf sections of plants were below the limit of detection regardless of treatment and sampling day.

### *3.2.2 Plant sections positive for S. Newport internalization in high, mid, and low treatments*

For plants transplanted into the high inoculated soil treatment, 35/36 (97.2%) of the root sections were positive for *S. Newport* internalization following plating and enrichment over the four sampling days (Table 5.2). Half (18/36) of the root sections for plants in the mid inoculated soil treatment were positive for *S. Newport* and varied between sampling day (4/9, 5/9, 7/9, and 2/9 for 1, 2, 3, and 7 d post-transplanting respectively). No root section samples were positive for *S. Newport* internalization from plants transplanted into the low inoculated soil treatment. There were no significant

differences ( $P > 0.05$ ) in *S. Newport* internalization in the root sections between sampling days for all three treatments. For each sampling day, except for day 2, the root sections from plants in the high treatment had significantly ( $P < 0.05$ ) more cases of *S. Newport* internalization than the other two treatments, while the root sections from plants in the mid treatment had significantly more cases of internalization than the low treatment. On day 2 there was no significant difference ( $P > 0.05$ ) in the cases of *S. Newport* internalization in the root sections from plants in the high and low treatments. Overall, root sections in the high treatment had significantly ( $P < 0.05$ ) more cases of *S. Newport* internalization compared to the root sections in the mid and low treatments, and the root sections in the mid treatment had significantly ( $P < 0.05$ ) more cases of internalization compared to root sections in the low treatment.

Of the stem sections sampled in the high inoculated soil treatment, 6/36 (16.7%) were positive for *S. Newport* internalization following plating and enrichment and positive stem samples varied between sampling day (2/9, 2/9, 2/9 and 0/9 for 1, 2, 3, and 7 d post-transplanting respectively) (Table 5.2). No *S. Newport* internalization was observed in the stem sections of plants in the mid and low treatments. For each sampling day the stem sections from plants in the high treatment had significantly ( $P < 0.05$ ) more cases of *S. Newport* internalization than the other two treatments, except for day 7 where no internalization cases were observed in the stem across all treatments. Overall, stem sections in the high treatment had significantly ( $P < 0.05$ ) more cases of *S. Newport* internalization compared to the root sections in the mid and low treatments. No cases of *S. Newport* internalization were observed in any of the leaves of the plants sampled across all sampling days and all three treatments.

#### 4. Discussion

Previous studies have determined that lateral roots may be a point of colonization for *Salmonella* and other human foodborne pathogens (Cooley et al., 2003; Karmakar et al., 2018). Results from this study support these findings as the roots of both the damage and intact treatments had high levels of *Salmonella* internalization in the roots. Though lateral roots may be a point of colonization, root damage facilitates the movement of the pathogen throughout the rest of the plant. Internalization of *Salmonella* increased and was significantly ( $P<0.05$ ) higher in stems of plants with damaged roots than plants with intact roots, suggesting that when roots are damaged *Salmonella* is given access to vascular tissue, such as the phloem and xylem, providing the pathogen opportunity to move into the stem and leaves of the plant. Due to the *Salmonella* size (0.5-1.5 microns wide and 2-5 microns long) (Andino and Hanning, 2015) and the size of the xylem and the phloem, pathogen movement through the xylem (50-160 micron) (van der Schoot and van Bel, 1989) of the plant is more likely than the phloem which contains sieve plates (1.5 micron) which inhibits the movement of particles in the plant (Mullendore et al., 2010).

Though, in a study performed by Guo et al., root damage did not seem to influence the internalization and movement of *Salmonella* in tomato plants grown hydroponically in an inoculated nutrient solution (Guo et al., 2002), which suggests that internalization may be dependent on plant species. More research is needed to determine what degree of root damage influences the movement of *Salmonella* into the aerial sections of the plants, such as the stem and leaves. Furthermore, plants that had the root stalk removed had significantly ( $P<0.05$ ) higher levels of *Salmonella* internalization in

the stem and leaves compared to plants with the root stalk (both intact and damaged root treatments). These results suggest that the root stalk has a valuable role in creating a barrier for foodborne pathogens to move into more susceptible sections of the plants such as the stem, leaves, and in extreme circumstances the fruit.

The results of this study suggest that colonization and internalization of *Salmonella* in the roots of bell pepper plants is highly dependent on the population level present in the soil during transplanting. The number of internalization events from pepper plants transplanted into the mid contaminated soil in this study are fairly comparable with the results presented in Chapter 4; of the tomato plants transplanted into inoculated soil at  $4.3 \log \text{CFU/g}$ , though internalization levels in the roots were higher for the transplanted tomato plants in chapter 4. This could be due to the physiological differences between tomato and pepper plants or varying environmental conditions between the two studies. Similarly, in a study performed by Beauchat et al., internalization was only observed in the roots of transplanted tomato plants exposed to soil inoculated with *Salmonella* ( $4.8 \log \text{CFU/ml}$ ) (Beuchat et al., 2003).

The roots of the peppers plants transplanted into the high inoculated soil treatment had all but one root sample positive for *Salmonella*, while the pathogen did not appear to move into the root section of the plant at such a high rate with only 6 of the 36 stem sections sampled being positive for *Salmonella* across all four sampling days. In a study performed by Hintz et al., internalization of *S. Newport* was observed in all the roots of plants irrigated weekly with inoculated water at  $7 \log \text{CFU/ml}$  at 14 days, while no stems were found positive for *S. Newport* internalization (Hintz et al., 2010). Likewise, in a study performed by Miles et al., only the roots of tomato plants administered

contaminated *Salmonella* Montevideo inoculated water (7 log CFU/ml) were positive for internalization (Miles et al., 2009).

Though *Salmonella* movements beyond the stem did not occur in the bell pepper plants after 7 days, consistent exposure to *Salmonella* over the plant's development may influence *Salmonella* movement into other sections of the plants. For tomato plants exposed to high concentrations of 7-8 log CFU/ml following transplanting internalization was observed in the roots, stem, leaves, and even the fruit (Hintz et al., 2010; Pollard et al., 2014; Zheng et al., 2013). More research is needed to understand the risk of internalization and transfer of *Salmonella* in bell pepper plants when exposed to *Salmonella* until flowering and fruit set. No translocation of bacteria to the fruit of bell pepper plants transplanted into soil inoculated with high concentrations ( $10^7$  CFU/g) of non-pathogenic *Escherichia coli* and *Listeria innocua*, though internalization of the bacteria was observed in the roots of tomato plants (Ganeshan, 2015), suggesting that the internalization and translocation of human pathogens from the roots to the fruit of bell pepper plants is unlikely.

## **Conclusions**

Due to the results of this study, findings suggest lateral root damage and *Salmonella* contamination levels may influence the internalization and movement of *Salmonella* in bell pepper plants. Though internalization events may be unlikely, proper care should be taken to avoid root damage of transplanted pepper plants along with the implementation of best practices to reduce the load of *Salmonella* in the soil in order to prevent internalization and transfer events from occurring.

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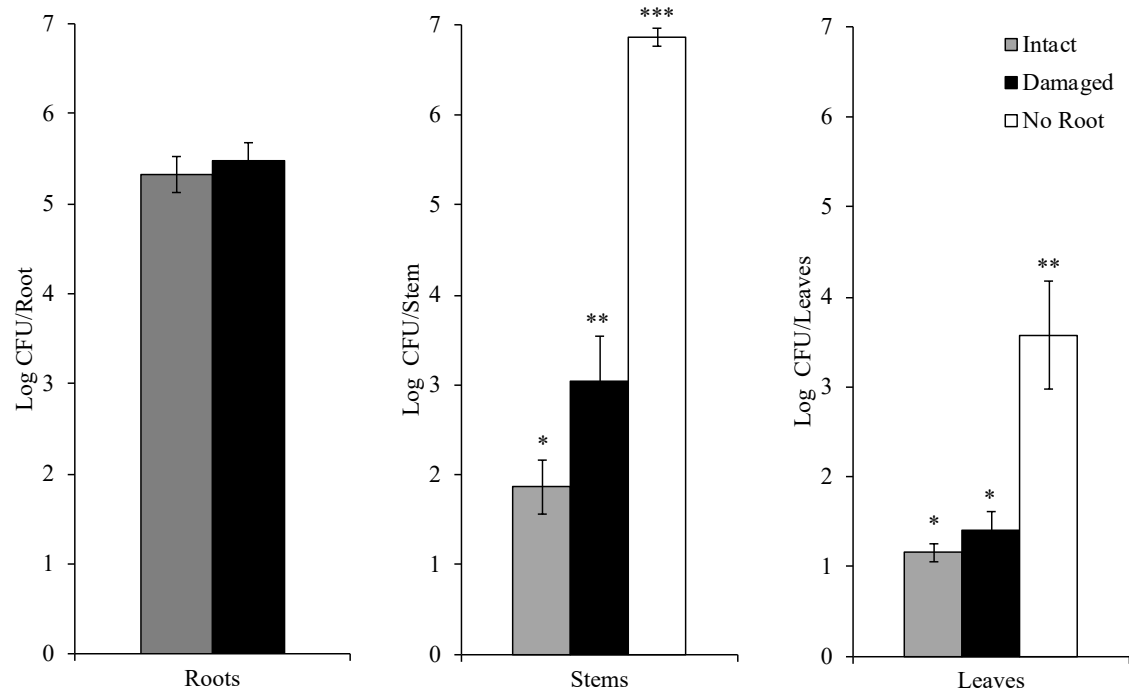
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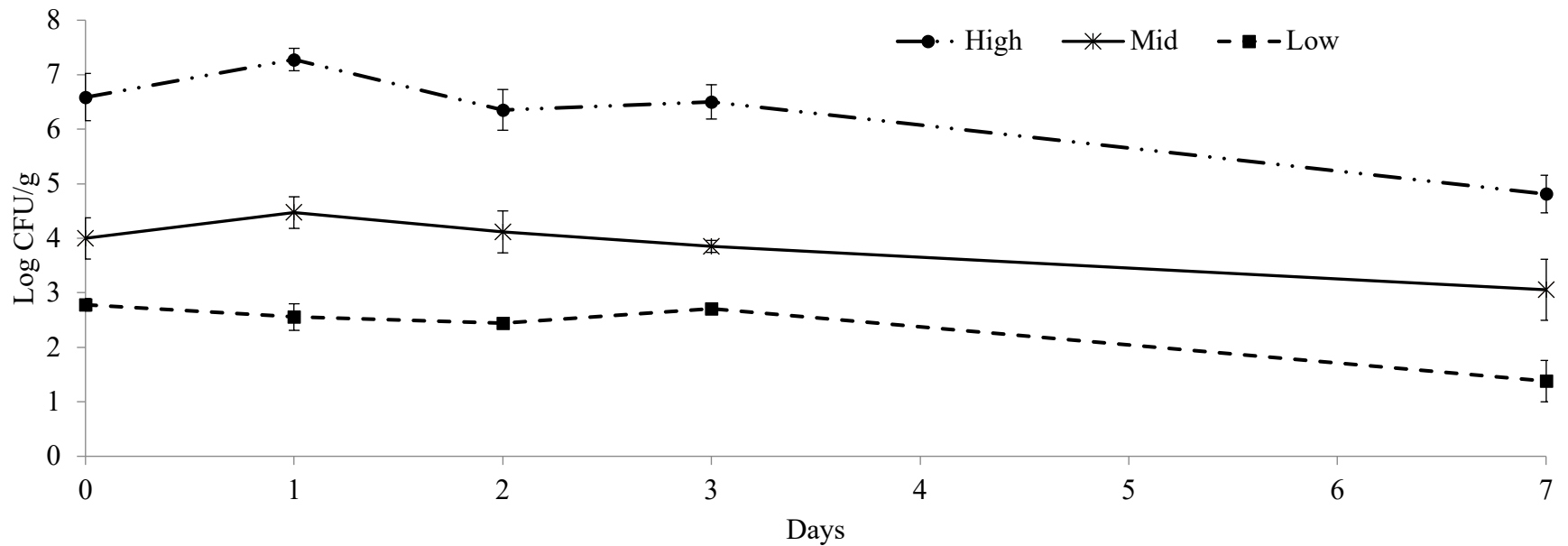
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## Figures



**Figure 5.1** Recovery of internalized *S. Newport* populations (log CFU/plant section) in the root, stem, and leaves of pepper plants in the three treatments; intact roots, damaged roots, and no root stalk. Error bars represent the standard error of the mean and \* represent significant differences ( $P < 0.05$ ) between treatments of a given plant section.



**Figure 5.2** Behavior of *S. Newport* population's (log CFU/g) in transplanted soil at varying inoculation levels (High, Mid, and Low) over the 7-day sampling period. Results shown are counts from XLD-N. Values are the average of one sample from each of the three replications (n=3), error bars represent standard error.

## Tables

**Table 5.1** Internalization levels of *S. Newport* in pepper plants 1, 2, 3, and 7 d following transplanting based on plant section and treatment

Day	Roots			Stem			Leaves		
	High	Mid	Low	High	Mid	Low	High	Mid	Low
Day 1	2.2±0.3 <sup>a</sup> A <sup>b</sup>	0.7±0.1 B	<0.7 B	0.8±0.1	<0.7	<0.7	<0.7	<0.7	<0.7
Day 2	1.8±0.2 A	0.7±0.1 B	<0.7 B	0.8±0.1	<0.7	<0.7	<0.7	<0.7	<0.7
Day 3	2.4±0.3 A	1.2±0.2 B	<0.7 B	0.9±0.2	<0.7	<0.7	<0.7	<0.7	<0.7
Day 7	1.9±0.3 A	0.8±0.1 B	<0.7 B	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7

<sup>a</sup> Represented as log CFU/plant section (i.e. root, stem or leaves) ± standard deviation, based on the average of three replications performed in triplicate samples (n=9) from counts obtained from XLD-N plates.

<sup>b</sup> Letters determine significance between treatments (High, Mid and Low) between roots ( $P \leq 0.05$ ).

<sup>c</sup> Below the limit of detection at <0.7 log CFU/plant section.

**Table 5.2** Positive pepper plant sections 1, 2, 3, and 7 d following transplanting based on treatment

Day	Roots			Stem			Leaves		
	High	Mid	Low	High	Mid	Low	High	Mid	Low
Day 1	9/9 <sup>a</sup> A <sup>b</sup>	4/9 B	0/9 C	2/9 A	0/9 B	0/9 B	0/9	0/9	0/9
Day 2	8/9 A	5/9 A	0/9 B	2/9 A	0/9 B	0/9 B	0/9	0/9	0/9
Day 3	9/9 A	7/9 B	0/9 C	2/9 A	0/9 B	0/9 B	0/9	0/9	0/9
Day 7	9/9 A	2/9 B	0/9 C	0/9	0/9	0/9	0/9	0/9	0/9
Total	35/36 A	18/36 B	0/36 C	6/36 A	0/36 B	0/36 B	0/36	0/36	0/36

<sup>a</sup> Values based on presence/absence of *S. Newport* following enumeration and enrichment between samples performed in triplicate from three replications (n=9).

<sup>b</sup> Letters determine significance between treatments (High, Mid and Low) between roots and stem ( $P \leq 0.05$ ).

## Chapter 6: Conclusion

*Salmonella* contamination of fresh produce at the preharvest level is a constant concern. Various modes of contamination exist, and proper steps should be taken to limit the potential risks of *Salmonella* contamination. Modes of *Salmonella* contamination of fresh produce at the preharvest level such as, irrigation water, soil, and biological soil amendments of animal origins present a concern because fresh produce never undergoes a kill-step to eliminate potential pathogen presence. Outbreaks of *Salmonella* Newport linked to fresh produce grown on the Eastern Shore of Virginia resulted in immense economic losses. Due to the burdens faced by the produce industry following a *Salmonella* outbreak, understanding sources of contamination at the preharvest level is vital in mitigating potential contamination. At the preharvest stage of production, amended and non-amended soils and irrigation water are likely sources of contamination.

In order to identify potential mitigation strategies, the objective of this dissertation was to determine potential routes of *Salmonella* contamination at the preharvest level for solanaceous crops and identify factors that contribute to potential *Salmonella* internalization. In order to accomplish this objective three studies were conducted to (i) evaluate how factors such as serovars, amendments, irrigation regime, and soil type influence the survival of *Salmonella* in agricultural soils ; (ii) determine the influence of varying modes of contamination (soil and irrigation water) on the internalization of *Salmonella* in transplanted tomato plants; and (iii) identify the roles root damage and *Salmonella* soil population have on the internalization of *Salmonella* in transplanted bell pepper plants.

In the first study, factors such as strain, soil type, irrigation regime, and the application of poultry litter were determined to be significant factors that contributed to the survival of *Salmonella* in agricultural soils. Strain was the most important factor in determining *Salmonella* survival in poultry litter amended sandy loam and clay loam soils, as growth and survival curves varied between the strains tested. Our models determined that Braenderup, Meleagridis, and Newport F as strains that had a significantly lower die off rate compared to the other strains tested. When assessing the risk of *Salmonella* survival in poultry litter amended agricultural soils, strain variability should be taken into consideration along with the interaction of other variables such as soil type and irrigation regime.

Soil moisture by irrigation regime, was found to influence the persistence of *Salmonella* in poultry litter amended sandy loam and clay loam soils. Strains in poultry litter amended soils irrigated weekly survived on average approximately 125 days longer than poultry litter amended soils irrigated daily. Though strains survived longer in soils irrigated weekly, population levels were generally lower in weekly irrigated soils compared to population levels in daily irrigated soils. The extent of survival of strains in soils that were irrigated weekly varied on strain and soil type. Varying intervals to maintain soil moisture heavily influenced the results of this study and should be considered heavily when assessing risk.

Results of this study found that the application of poultry litter influenced most strains in two ways; (i) promoting significant growth and (ii) significantly increasing the persistence of *Salmonella* regardless of soil type. In some cases, *Salmonella* strain populations increased as much as 2 and 1.5 log CFU/g in poultry litter amended sandy loam and clay loam soils, respectively. Based on our model the application of poultry litter would increase *Salmonella*

populations by an estimated 1.72 log CFU or MPN/g. In our study, the application of poultry litter increased the survival of *S. Newport E* by 56 and 112 days in sandy and clay loam soils respectively. Measures should be taken to ensure that safe intervals are determined when apply untreated poultry litter to ensure that the risk of fresh produce contamination is mitigated.

Thus, in order to assess the risk of *Salmonella* survival in soil, poultry litter application, soil moisture/irrigation, soil type, and strains associated with the area being assessed, should be highly considered. The United States Department of Agriculture's (USDA) National Organic Program (NOP) has determined that intervals for applying un-treated, raw manure are 120 days between application (incorporation) and harvest for produce that has its edible portion directly in contact with the soil, or 90 days for produce that does not have its edible portion in contact with the soil. For the most part the results from this study support these determinations, though certain strains survived much longer than 120 day following amendment application based on soil type and irrigation regime. The data reported here can be used to support risk assessments; as well as, assist risk managers in evaluating the likelihood of long-term *Salmonella* survival (using worst case scenario strains) and application of poultry litter application to agricultural soils.

In the second study, the internalization of *Salmonella* in the roots of transplanted tomato plants was found to be heavily influenced on the mode of contamination. Plants transplanted into contaminated soil had higher internalization events in the roots than transplanted plants irrigated with contaminated irrigation water. The results of this study suggest that plants exposed to a single contamination event via irrigation water do not present a risk of internalization occurring through the roots. Though internalization occurred in tomato plants transplanted in contaminated soil or repeatedly irrigated with contaminated irrigation water, there was no evidence of transfer

from the roots to the fruit of the plant. These results suggest that the internalization of *Salmonella* in the roots and subsequent transfer to the fruit is unlikely, though measure should be taken in order to ensure that contamination of produce doesn't occur via contaminated soil or irrigation water.

Results of the third study suggest that the uptake and movement of *Salmonella* in bell pepper plants was highly dependent on root conditions and population levels present in the soil. Though the internalization of *Salmonella* in the roots of pepper plants with damaged roots weren't different compared to plants with intact roots, significant *Salmonella* movement to the stem was observed in plants with damaged roots. These results suggest that when roots are damaged or removed *Salmonella* is given greater access to vascular tissue, and particularly the xylem, providing the pathogen opportunity to move into the stem and leaves of the plant. Furthermore, results suggest that the root stalk is vital in creating a barrier for foodborne pathogens to move into more susceptible areas of the plants such as the stem, leaves, and fruit. Measures should be taken to ensure that root damage is avoided when transplanting.







In plants transplanted into soils with high populations (6-7 log CFU/g) of *Salmonella* had significantly higher internalization events occur than plants transplanted into soils with medium (4-5 log CFU/g) and low (2-3 log CFU/g) *Salmonella* populations. However, internalization events did occur in plants transplanted into soil with medium *Salmonella* population levels, while no internalization events occurred in plants transplanted into soils with low *Salmonella* population levels. These results suggest that actions should be taken to reduce *Salmonella* populations to < 3 log CFU/g in soil in order to prevent the risk of potential internalization.

These studies further our understanding of the survival of *Salmonella* in poultry litter amended soils and the potential for internalization of the pathogen through the roots in tomato and bell pepper plants. These results may be used to implement best practices in order to reduce the potential internalization and contamination events of fresh produce during the preharvest stage of production. Strategies in order to limit or reduce root damage during transplanting and the presence of *Salmonella* populations in agricultural soils and irrigation water should be implemented in order to reduce contamination and potential internalization of the pathogen.

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