OBSERVING *SALMONELLA* INTERNALIZATION FROM CONTAMINATED SEEDS AND IRRIGATION WATER IN GREENHOUSE TOMATOES

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Abstract

Greenhouse grown tomato fruits and tissues were tested for the presence of *Salmonella* after the plants had been treated with *Salmonella* contaminated irrigation water or grown from contaminated seeds. Greenhouse grown tomato plants were placed into eight different groups. Groups one through six consisted of five plants each and were treated with 350 ml of 10^6 *Salmonella* contaminated irrigation water over a course of 70 days; group one received one 350 ml 10^7 *Salmonella* treatment, group two received two treatments, and so on, the treatments were scheduled every 14 days. Group seven was the control that consisted of five plants and received no *Salmonella* treatment. Group eight was grown from seeds that had been contaminated with *Salmonella* by soaking the seeds in a 10^8 *Salmonella* suspension for 24 hours at room temperature, and received no *Salmonella* watering treatment. A total of 128 tomatoes were sampled from the tomato plants of all three groups and none tested positive for *Salmonella*. Tissue samples consisting of roots, leaves, and stems, and were collected from one plant per each of three replications. No leaves or stems contained *Salmonella*, however, five of the twenty-four root samples were positive for *Salmonella*.

In a second study, *Salmonella* was tested for its ability to survive in three concentrated fertilizer stock solutions and 1.6% diluted solutions of the fertilizer. Fertilizer sample CF-S was a stock solution of commercial 20N-4.4P-16.6K fertilizer, US-S was a mix of 11.3 kg UltraSol, 4.5 kg Epsom Salts, and 2.3 kg 0N-0P-43.2K in 114 L water, Fertilizer CN-S is a mix of 11.3 kg Calcium Nitrate and 56.7 g Iron chelate (10%) to 30 L water; Fertilizers CF-1.6, US-1.6, and CN-1.6 were the 1.6% fertilizer dilutions respectively. There was no significant difference ($p<0.05$) between the survival of *Salmonella* in fertilizer groups CF-1.6, US-S, US-1.6, CN-1.6, and the sterile distilled water control; all but US-S yielded less than a one log reduction in *Salmonella* over a period of 72 hours. US-S yielded over a two log reduction in *Salmonella* and was not significantly different than CN-S which had over a four log reduction. CF-S was significantly different than all samples and led to over a 6 log reduction of *Salmonella*.

The results of this study showed no evidence that *Salmonella* was able to internalize in Cultiver trust tomato fruit or tissues above the root line when irrigated with contaminated water into the pine medium under greenhouse conditions. There was also no evidence that *Salmonella* is able to internalize in any tissues or fruit from contaminated seeds. The results also show that *Salmonella* was not able to survive in the commercial fertilizer stock solution (CF-S), and had limited survival in CN-S tomato fertilizer solution. The diluted fertilizer solution and US-S stock solution showed no significance in survival of *Salmonella* when compared to the sterile water control.

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Literature review

Salmonella Review

Salmonella is one of the leading causes of foodborne illnesses in the United States, it is estimated to have caused 1.5 million cases of infection, 15,000 hospitalizations, and 500 deaths annually *(22, 35)*. Although the infective dose of *Salmonella* is not known, it is estimated that ingestion of only a few cells may cause salmonellosis. Salmonellosis is a typically mild gastrointestinal illness whose symptoms include fever, nausea, vomiting, diarrhea, and abdominal cramping *(30, 36)*. These symptoms typically only last one to two days.

Salmonella is a gram negative, small rod shaped, facultatively anaerobic, bacterial pathogen that belongs to the family enterobacteriacia. *Salmonella* are typically motile organisms with peritrichous flagella, however there are nonmotile serovars such as *S*. Pullorum and *S*. Gallinarum that do not have flagella, as well as strains with dysfunctional flagella that are also nonmotile *(36)*. Another characteristic of *Salmonella* is the ability to utilize an acidification tolerance response (ATR). *Salmonella* are neutrophilic and therefore able to grow over a wide pH range of about pH 5 to 9. When exposed to extreme conditions the cell is able to undergo ATR. This mechanism allows the organism to withstand extreme acidic pH (pH 3.0-4.0) if first exposed to a mild acidic condition (pH 5.5-6.0) known as preshock. Preshock occurs through ATR pH homeostasis mechanisms such as Na+/H+ and K+/H+ antiporters. Acid shock is the second exposure to an extreme acid conditions (below pH 4.5) that triggers a different set

of proteins that protect against extreme low pH; however, the cell would not be protected without the initial preshock ATR mechanism. Cells with this first exposure have a 100-1,000 fold increase chance of survival than unadapted cells *(18)*.

Salmonella is an enteric pathogen and therefore its sources typically originate from a human or animal carrier. Carriers of *Salmonella* include farm animals, birds, reptiles, humans and sometimes insects, because it is commonly found in the intestinal tract of these organisms. Being an intestinal organism allows for bacteria to reenter the environment through feces and then be carried by insects, or possibly to contaminate polluted waters *(30)*. Typical foods associated with *Salmonella* include animal products such as eggs, dairy, poultry, and meat, but outbreaks have more recently been linked to fresh fruits and produce *(22)*.

The consumption of fresh fruits and vegetables has risen in recent years. This may be due to a more health conscious society as well as research that correlates the increased consumption of fresh fruits and vegetables with decreased risk of stroke, high blood pressure, and cardiovascular disease *(27)*. This increase in consumption has led to an increase in produce related outbreaks. It is estimated that in the 1970's produce related outbreaks averaged 2 per year, and in the 1990's this number has risen to an average of 16 per year. A study done by the CDC analyzed a total of 190 produce related outbreaks between the years 1973 and 1977 *(45)*. Of these outbreaks *Salmonella* was responsible for 31 (48%), which was the highest occurrence for the bacterial pathogens. The most common serotypes reported were Typhimurium (5 outbreaks), Montevideo (4), and Javiana (3), and a total of 20 serotypes were reported to be the source of at least two outbreaks *(45)*.

In a study completed by Samadpour et al. *(43)* samples of ground beef, bean sprouts and mushrooms that had been collected from retail food stores, were tested for presence of EHEC, *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. Of the 200 samples of ground beef and sprouts and 100 samples of mushrooms tested, 67 (4.2%) ground beef, 14 (7%)sprouts, and 5 (5%) mushrooms tested positive for *Salmonella (43)*. The study was particularly important in showing the presence of pathogens in food that were likely to be consumed raw.

Detection of Outbreaks

Two epidemiological methods used to determine *Salmonella* outbreaks include *Salmonella* Outbreak Detection Algorithm (SODA) and PulseNet. SODA is a computer algorithm used to detect clusters of *Salmonella* cases reported by the Public Health Laboratory Information Systems (PHLIS) *(44)*. The algorithm uses an average number of illnesses reported over the past five years as its baseline, and then compares any unusual amount of cases to the baseline. PulseNet is a network of public health laboratories that collect pulsified gel electrophoresis (PFGE) results used for disease surveillance of foodborne pathogens. They currently record molecular subtyping of *Campylobacter jejuni, Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, and *Shigella (12)*. These results are sent to a database at the Center for Disease Control and Prevention where they are monitored for clusters of PFGE patterns *(12, 44, 49)*.

Plant pathology

The first interactions between a plant and the microorganism are the most important; it is in this time period that determines the microorganisms ability to internalize. Typically the plants natural defenses are able to repel microorganisms, however, these defenses are not always successful *(48)*. With correct conditions bacteria are able to enter a plant's leaves through the stomata *(48)*, as well as enter the fruit through the leaf, flower, and stem scar *(34)*. However, plants such as tomatoes contain many secondary metabolites such as α-tomatine that may have antimicrobial properties and make survival on fruit leaves much lower than on the fruit itself *(40)*.

Fruits and vegetables are particularly susceptible to microorganisms when their natural structure is damaged and microorganisms are able to enter through wounds, cuts, or punctures *(17)*. Roots wounded naturally through growth also provide another major point of entry for bacteria *(23)*. Bacterial soft rot has also been associated with the increased incidence of *Salmonella* in produce. Wells and Butterfield *(53)* compared the incidence of *Salmonella* in healthy fruits and vegetables compared to those with soft rot. They found that the healthy vegetables had an incidence of 9 to10% *Salmonella* contamination, and the soft rot vegetables had an incidence of 18 to 20%. They also tested the incidence of *Salmonella typhimurium* inoculated on potato, pepper, and carrot disks, that have been inoculated with either *Pseudomonas viridiflava* or *Erwinia carotovora*; they found *Salmonella* at a 10 times higher concentration when co-inoculated with *E. carotovora* and a 3 times higher concentration when co-inoculated with *P. viridiflava.*

Beuchat and Ryu *(4)* discussed the pre-harvesting practices and likely contamination points for human pathogens to fresh fruits and vegetables. Common pre-harvest sources include domestic animals, insects, feces, irrigation water, and human handling. Animals that particularly contribute to the contamination of produce in the field are birds and reptiles that are known carriers of *Salmonella*. Another common cause of contamination of the field is runoff from neighboring livestock farms that may contaminate irrigation water.

Past Outbreaks of Salmonella Associated with Produce

Mangoes

In December 1999 the CDC detected a rise in *Salmonella* Newport cases that were later attributed to mangos. Out of a total of 78 cases, 28 patients enrolled in a case study that found 50% of them consumed mangoes within the last five days, compared to 10% of the controls. The investigation identified a single Brazilian farm as the source of the outbreak *(44)*. The source of contamination was traced back to a hot water treatment used for the prevention of tephritid fruit flies to be brought inside United States borders. The hot water disinfestations treatment is used on all exported mango fruit to the United States and Japan, and is currently the only authorized treatment. The fruit is placed in a hot water bath of 46.1° C for 65, 75, or 90 minutes depending on fruit weight. This is then followed by a cool water bath of 21° C for 6 to 10 minutes. The cool water is treated with chlorine at a level of 100 mg/liter, and the cool water was reused for 1 week unless it becomes excessively turbid *(38, 44)*.

Previous Studies

Penteado et al. *(38)* studied the internalization of *Salmonella* in mangos using a stimulated postharvest disinfestation process like the one explained above. The mangos were from a Florida producer so there was no need for disinfection for fruit flies. The study demonstrated internalization by using a dye and a pathogen uptake study. The 22° C water tank was inoculated with either 0.1% brilliant blue FD&C no. 1 for the dye uptake study and 10^7 *Salmonella* for the pathogen study. The dye study showed that 10 of 15 mangos were positive for dye internalization, however the internalization only occurred directly through the stem scar tissue and into the center of the fruit; the side and bottom segments had no presence of dye, showing the route of internalization through the stem scar tissue and only through the center of the fruit. The pathogen study resulted in 83% of the mangos containing *Salmonella* in the stem end. The results of this study demonstrated the potential of *Salmonella* to enter the fruit during the post harvesting processes and emphasizes the importance of water quality during these practices.

Tomatoes

Outbreaks

Within recent years tomatoes have been a common vehicle for Salmonellosis outbreaks. Tomatoes are a large produce commodity in the United States, with approximately 5 billion pounds eaten yearly *(11)*. In the early nineties there were two major tomato related Salmonellosis outbreaks. In 1990, 176 cases of *Salmonella* Javiana were reported in a multistate outbreak. Another outbreak followed in 1993 when 100

cases of *Salmonella* Montevideo were traced back to tomato consumption. Both of these outbreaks were then traced back to a single packinghouse in South Carolina *(24)*. In 1998 a multistate outbreak of *Salmonella* Baildon was traced back to restaurant serving raw tomatoes. The strain was recovered from 86 patients over a three week period ending in January 1999. The source of contamination was likely to have occurred on the farm or during packing *(13)*. In July 2002 the Minnesota Department of Health identified two cases of *Salmonella* Javiana that were later traced back to the Transplant Games in Orlando Florida *(46)*. A web-based investigation was initiated and both athletes and spectators who had attended the games were asked to complete an electronic survey about the outbreak. Survey participants were asked to complete a second survey specifically detailed to the food the participants consumed. The web-based investigation surveyed a total of 1,100 attendees, 369 of which responded and found 82 reported illnesses *(46)*. There are more possible cases of *Salmonella* than those reported because not all of the attendee's email addresses were available, and not all those surveyed responded.

In the summer of 2004 there were three outbreaks of *Salmonella* associated with Roma tomatoes in the United States and Canada. These outbreaks caused 561 outbreak related cases in eighteen states and one providence of Canada. The FDA traced back two of the outbreaks to a single packinghouse in Florida, however, other suppliers and post harvesting facilities may have added to the possibility of contamination. The multistate cases in only the US yielded multiple serotypes, however the second two outbreaks yielded Braenderup in the multiple U.S. states and Javiana in Canada *(11)*.

Previous studies

The survival of *Salmonella* on tomato leaf surfaces is important because of the possibility of the bacteria entering the plant through the stomata on the leaves, or even contact of the leaves with the fruit during harvesting. *Salmonella* Montevideo spiked on tomato leaf surfaces was able to survive for at least 6 days *(40)*. Rathinasabapathi *(40)* also tested the effect of ethylene treatments used for fruit ripening on the survival of *Salmonella* on the surface of mature green tomatoes. The ethylene treatment had no significant effect on the survival of *Salmonella* on the fruit surface *(40)*.

Guo et al. *(23)* demonstrated the ability of *Salmonella* to internalize in hydroponically grown tomato seedlings with a contaminated nutrient solution. There was evidence of *Salmonella* internalization in hypocotyls and cotyledons, stems, and leaves after 24 hours (3.01, 3.40, <1.22 CFU/g respectively) of exposure and reached higher levels after 9 days (4.02, 3.70, and 3.61 CFU/g) of root uptake *(23)*.

Other studies have demonstrated *Salmonella*'s ability to attach and survive on the surface of tomato fruit. Iturriaga et al. *(28)* studied the effect of relative humidity, storage temperature, and ripening stage on attachment of *Salmonella*. They found that a combination of temperature and relative humidity had a significant effect on the *Salmonella* attachment to the fruit *(28)*. Guo et al. *(21)* tested the survival of *Salmonella* in tomato plants after inoculation by brushing the flowers with inoculum as well as injecting the stems. Twenty five percent of the fruit from inoculated flowers was positive for *Salmonella*, while forty and forty three percent of fruit was *Salmonella* positive from stems that had been inoculated pre and post flowering respectively *(21)*. Insects that may be attracted to the flowers could serve as a vector and contaminate the blossoms which this study demonstrated could then lead to a contaminated fruit.

Other studies have examined the possibility of plant parasites aiding in the internalization of other pathogens in the tomato plant. Parasitic plant nematodes such as *Meloidogne incognita*, create wounds in the roots when they colonize and feed off the plant. Beuchat et al. *(5)* studied the effect that the presence of *M. icognita* has on the internalization of *Salmonella* in tomato plants by coinoculating the organisms in the soil of tomato plants. They found that no evidence that *M. icognita* facilitates *Salmonella* internalization in tomato plants *(5)*.

Pesticide solutions may also be a vector of transporting *Salmonella* as well as other pathogens to tomato plants. Guan et al. *(20)* tested the survival of *Salmonella*, *E. coli*, *Shigella*, and *Listeria monocytogenes* in seven different pesticide solutions sprayed on tomato plants. They found *Salmonella* survived best out of the tested microorganisms and was recovered along with *E. coli* on the leaves and fruit skins of harvested fruit that had been sprayed with Bravo 500 fungicide solution.

Disinfecting washes are a common post-harvest control of microorganisms on tomato fruit, however, they cannot always affect the possible internalization of *Salmonella*. Ibarra-Sanchez et al. *(27)* tested the internalization of *Salmonella* that had been dipped or sprayed (there was no significant difference between the two inoculation techniques) and then treated with rinsing in tap water, hypochlorite solution, or lactic acid solution (2% wt/vol.). Both the lactic acid and chlorine treatments showed a significantly decreased surface population that was below detectible limits for all but two treatments (lactic acid and hypochlorite dips at 5° C). Tomatoes treated with the lactic acid spray recovered no *Salmonella* from all but one sample *(27)*. *Salmonella* is susceptible to both

lactic acid and chlorine treatments, however, cells that may have seeped into the tomato previously would be able to evade the antimicrobial treatments.

Seed Sprouts

Sprouted seeds that are harvested shortly after germination are typically consumed raw and have been a vector of many foodbourne illnesses. There are a variety of sprouts grown from different plants such as alfalfa, mung bean and wheat. Spouts are a large commodity in the United States. The International Sprout Growers Association estimates that there are currently 475 growers in the United States that produce approximately 250 million dollars worth of food sprouts each year *(50)*, this emphasizes the importance of sprout producers using proper control methods. The possibility of contamination by human pathogens to sprouts is increased by the sprouting process, where they are placed in a warm humid environment for 3 to 7 days *(19, 52)* Many foodborne outbreaks have been traced back to sprouts as the source, mainly caused by the pathogens *E. coli* and *Salmonella*.

These outbreaks let to the Food and Drug Administration to establish extra guidance for sprout growers. The FDA identified several points that may need to be controlled in sprout production including following good agricultural practices (GAPs) and good manufacturing practices (GMPs) for the handling of sprouts and seeds, seed disinfection treatments and microbial testing of the product before it enters the food supply *(16)*. Microbial testing should be done on spent irrigation water for the presence of pathogens. This is done so that water is tested for the presence of a pathogen before the product is shipped and distributed *(17, 39)*. Following FDA guidance as well as

Good Agricultural Practices are effective measures in preventing sprout related foodborne outbreaks.

Outbreaks

In 1995 two separate multinational outbreaks of *Salmonella* occurred that had been traced back to seed sprouts *(19)*. The 1995 outbreak of *Salmonella* Stanley occurred in the United States and Finland from alfalfa sprouts grown from contaminated seeds *(33)*. The second outbreak of *S*. Newport during December 2005 was restricted to Oregon and British Columbia *(51)*. These two outbreaks brought to attention sprouts as a vector of foodbourne disease.

In 1999 the Oregon Department of Human Services (ODHS) conducted an investigation to trace back cases of *Salmonella* Mbandaka, a rare serotype that occurred of at a rate of 1.5 cases per year in Oregon. ODHS detected a multistate outbreak with 87 confirmed cases of the serotype throughout Oregon, Washington, Idaho, and California. The investigation traced the outbreak back to a single lot of seeds that was used by five sprout producers. Of the five producers, only two that did not use FDA recommended disinfecting procedures were linked to the illnesses *(19)*.

In September 1999 a multistate outbreak of *Salmonella* Muenchen occurred throughout seven states. A total of 157 outbreaks occurred, 65 of which were located in Wisconsin, however it was estimated that approximately 3,500 to 16,200 total cases occurred when accounting for cases not reported. The Wisconsin Department of Agriculture, Trade and Consumer Products (WDATCP) and the Wisconsin Department of Public Health released press statements advising against the sale and consumption of

sprouts, which proved to be effective at preventing further outbreaks. WDATCP and WDPH were later able to identify three sprout distributors and two sprouters as the source of the outbreak. The sprouters both followed the current FDA processing regulations of treating the sprouts with calcium hypochlorite. The FDA now also recommends that all spent irrigation water of sprouts be tested for *E. coli* O157:H7 and *Salmonella* spp. *(39)*.

Previous Studies

Warriner et al. *(52)* tested both *Salmonella* Montevideo and *Escherichia coli*'s ability to internalize in mung bean sprouts. They found when the pathogens were present on the outside of the seeds they had the ability to internalize within the sprouts. This study showed the importance of the control of sprout production, because the internalized bacteria then can not be controlled by any decontaminating washes.

Previous studies have tested the efficacy of different methods of seed disinfection. Most of the methods use a biocidal wash. Hu et al. *(26)* tested a heat treatment of mung bean seeds when germinated to sprouts. They found that a heat treatment of 55° C for at least 4 days was effective in lowering the levels of *Salmonella* and *E. coli*, without affecting the germination rate. However, previous studies have found this treatment to affect the rate of germination for alfalfa spouts so this treatment is not recommended for all types of seed sprouts *(26)*.

Melons

Throughout the last 20 years cantaloupes have been implicated in several *Salmonella* related outbreaks. The first happened in 1990 when an outbreak of *Salmonella* Chester linked to cantaloupes caused illness to 245 persons in 30 states. The following year *Salmonella* Poona was responsible for an outbreak with approximately 400 confirmed cases throughout 23 states and Canada. The cantaloupes were later found to have been distributed from Texas, although some may have come from Mexico *(9, 10)*. An additional outbreak with *Salmonella* Saphra occurred during 1997. This was a smaller outbreak with 23 cases and isolated to the state of California. These melons were also thought to be from Mexico *(10)*.

In the years of 2000 to 2002 there were three cantaloupe related *Salmonella* Poona outbreaks. The 2000 outbreak consisted of 47 confirmed cases across six different states. The 2001 outbreak was first identified when a California patient was diagnosed with a strain of *Salmonella* with the rare trait that it did not produce H_2S; a total of 50 cases were later identified across five states. The 2002 outbreak was identified over 11 states and four provinces of Canada. The FDA later traced the outbreaks back to shippers and farmers in Mexico and found possible points of contamination such as contaminated irrigation water, improper cleaning and cooling of fruit, poor worker hygiene, and pests in the facilities; the FDA provided guidance on proper procedures for production, packaging and shipping of produce *(3)*. *Salmonella* Poona the serotype identified for these outbreaks is often the cause of reptile contracted outbreaks and is otherwise rare. The CDC hypothesizes that these outbreaks may have then been caused by reptiles found

in the environment who were drawn to the fields and packinghouses to feed on the melons *(10)*.

Watermelons are not a common source of *Salmonella* infections, and have been linked to only three outbreaks in the last 60 years. The first occurred in 1950 in Michigan when *Salmonella* of an unidentified serotype caused six cases. The next outbreak did not occur until 1979, when 18 cases of *Salmonella* Oranienburg were confirmed in Illinois. The last known outbreak of *Salmonella* related to watermelon occurred in 1991, when a group of 12 school children were confirmed as contracting *Salmonella* Javiana *(6)*. The number of *Salmonella* outbreaks related to cantaloupes since 1990 indicated to the Food and Drug administration the need for better control in the growing, processing, and packing of cantaloupes.

Previous Sudies

Parnell et. al. *(37)* tested different methods to lower the pathogen counts on the outside of cantaloupes and honeydew melons. They found that *Salmonella* was typically found in lower amounts on the rinds of honeydew melons than cantaloupes, due to honeydews smooth waxy surfaces. They also found that wash water and scrubbing with brushes had the ability to transfer *Salmonella* from contaminated to uncontaminated surfaces. However, the use of sanitizers such as chlorine lowered the possibility of this transfer. Therefore they recommend that melons be scrubbed and effectively sanitized *(37)*.

The USDA tested the efficacy of surface pasteurization on cantaloupes surface-inoculated with *Salmonella* Poona. When the cantaloupes were treated at 76° C for 2 to 3

minutes they yielded at least a 5 log reduction of the number of *Salmonella*. The pasteurized cantaloupes also stayed firm and did not mold when held in storage, whereas the control samples became moldy and soft. The pasteurization proved to not only be an effective method in pathogen reduction but also an a good method in raising shelf life quality *(2)*.

Mold growth may also have an effect on the growth of *Salmonella* on cantaloupes. Richards and Beuchat *(41)* found that cantaloupe rinds containing molds *C. cladosporioides* or *G. candidum* raised the pH to provide a more beneficial environment for *Salmonella* to grow. To test the survival of *Salmonella* on cantaloupe rinds, *Salmonella* was inoculated on the rinds at a level of 3.3 log CFU/g. After a storage period of 14 days at 20° C they found the level of *Salmonella* to increase to that of about 9.5 log CFU/g. However, there was no significant difference in counts when a combination of *Salmonella* and mold species or only *Salmonella* was inoculated on the cantaloupe rinds, despite the more desirable pH on rinds with molds *(41)*.

Cilantro

In March 1999 an outbreak of a total 76 cases *Salmonella* serotype Thompson was found in the Los Angeles area. Typically the state of California has less than nine outbreaks per month, so the increase in cases spurred an investigation. The outbreak was later traced back to the consumption of cilantro, and most of the cases were traced back to restaurant prepared fresh salsa. Three of the five restaurants received the implicated cilantro from the same region of Mexico; however, the investigation was hampered by poor record keeping. This is the first outbreak of *Salmonella* attributed to cilantro,

although there has been a recent outbreak of *Shigella* related to cilantro, also received from Mexico *(8)*.

In a recent study *(7) Salmonella* was able to colonize on cilantro leaves after inoculation. The *Salmonella* grew best under warmer conditions (30°C) and a high relative humidity. The study was able to show the ability for *Salmonella* to survive on cilantro leaves through different environmental conditions and emphasized the potential of pre-harvest contamination causing illness.

Lettuce and Bagged Salad Products

Over the past few years an increasing number of *Salmonella* outbreaks related to lettuce and bagged salad product have occurred internationally. A study in the United Kingdom tested 3,852 samples of bagged prepared salad. They found that 99.3% of the salads were satisfactory and safe. Of the 36 samples found unsatisfactory, six were contaminated with *Salmonella (42)*.

In September 2000 an outbreak of *Salmonella* Typhimurium occurred in England and Wales. Three hundred and sixty one confirmed cases *S*. Typhimurium definitive phage type 104 were reported in August and September of 2000, of these 361 cases, 258 were tested under Pulsified gel electrophoresis and of those 174 shared a common plasmid profile. There was a strong correlation between illness and consumption of lettuce away from home, however there was no traceback to a common supplier or food outlet *(25)*.

In 2001, an outbreak of *Salmonella* Bovismorbifican phage type 32 occurred in Queensland, Australia. A total of 41 cases were identified, 36 of which were phage type

32 and tracedback to 15 different restaurants of a common fast food chain. The lettuce product was then traced back to a food processor where a sample taken from a lettuce shredder with food residue yielded *Salmonella* Bovismorbifican phage type 32 *(47)*.

Consumer Safety

Consumer awareness education is a very important factor for prevention of foodborne illness. In a survey, Li-Cohen et al. *(32)* found that 81% of consumers wash fresh produce before consuming, however, only 65% of consumers wash melons before consuming. The main reason for consumers not washing fruits such as melons, because they do not consume the outside skin or rind, as well as the consumer believed the produce was already clean *(32, 37)*. Consumers need to be aware that although the rind of some fruits may not be consumed, the outside may still need to be washed because bacteria present may be transmitted when the consumer cuts through the surface into the flesh.

Routes of Microbial Contamination

Pre-Harvest

Outside environments that fruits and vegetables are grown in provide many factors that may lead to potential contamination. Some proposed vectors of preharvest contamination include contaminated irrigation water, previously contaminated seeds, and living vectors such as birds, reptiles and insects. Irrigation is a necessary system to many farming operations, however, contaminated irrigation water has the potential to further contaminate a large portion of crop that it is used to irrigate. Irrigation water may be

contaminated with *Salmonella* from nearby livestock farms through rainwater runoff *(22)*, as well as birds and reptiles in the environment. Sewage contamination of irrigation water is also possible; *Salmonella* (as well as *Ascaris* ova, and *Endamoeba coli* cysts) was recovered from over half of irrigation water samples that had been contaminated by sewage *(4)*. Contaminated irrigation water can then become a vector to the produce by direct contact or internalization if under the right conditions. Birds, reptiles, and insects may also contaminate the fruits and vegetables through direct contact.

Post-Harvest

Post harvest points of control are very important because of the high quantities of fruits and vegetables that are processed. Therefore post harvest practices must be strictly regulated to avoid contamination. Packinghouses are frequently implicated as a sources of contamination. Line equipment for cutting, packing or other post-harvest processes must be properly cleaned and sanitized. Another important aspect of post-harvest control is temperature; temperature of produce must be kept within critical limits to control pathogen survival. Dump tanks and water baths may cause contaminating organisms on the outside of the fruit to transfer to the internal tissue through water*(4)*. Bruised or broken skins of the fruit act as an entry point for bacterial pathogens. This route of contamination can be controlled by using disinfecting treatments which will reduce numbers of the pathogen, however, these will only reduce contamination on the outside of the fruit and cannot decontaminate the internal tissue of the fruit *(27)*. Post-harvest practices should be strictly regulated to help prevent the spread of microbial contamination.

Points of Control

It is important for the produce growers and packinghouses to establish a Hazard Analysis and Critical Control Point (HACCP) plan. For growers an important HACCP point is control of irrigation water quality and insure proper testing is done. For packinghouses it is important to monitor the quality of water for the sanitizing baths for pH, chlorine levels, and water temperature *(11)*.

In a study done by Duffy et al. *(14)* irrigation water, packing shed equipment and fresh produce including cantaloupes, oranges, and parsley, were tested for the Salmonella isolates. Of 1,257 samples collected from two different farms over two seasons 26 were positive for *Salmonella*. Sixteen of these were isolated from irrigation water, six from packing shed equipment, and three from washed cantaloupes. Of the six isolates from packinghouse equipments, five were on surfaces that could come in contact with produce that was ready to be boxed and shipped without further processing *(14)*.

Allen et al. *(1)* tested the survival of *Salmonella* on the surfaces of a Florida packinghouse materials as well as the surface of tomatoes. Stainless steel, PVC, sponge rollers, and unfinished oak were the surfaces tested. The study showed that stainless steel, PVC, and the unfinished wood overall could support *Salmonella* survival over the fall and winter months but not over the summer. The sponge rollers did not show survival of *Salmonella*, however under certain conditions may be able to support survival by giving the bacteria a porous moist surface. Allen et al. *(1)* also found that *Salmonella* was able to survive for approximately 28 days on the surface of tomatoes left in

conditions comparable to those of the Florida spring production season, fall and winter production season, or a ripening room *(1)*.

Conclusion

Salmonella is one of the most common foodborne pathogens in the United States, and is estimated to cause millions of illnesses every year. Recent trends in illness have shown more outbreaks related to fresh fruits and vegetables; because these foods are readily consumed raw, precautions must be taken to help ensure produce safety. Further research must be done to study the mechanisms of how *Salmonella* and the food interact, as well as what points of control in the growing and harvesting processes are of most concern. Growers and packers of fresh fruits and vegetables must also pay special concern to GAPs and GMPs set in place for their business. Control needs to be taken because the growing consumption of fresh fruits and vegetables can lead to large scale *Salmonella* outbreaks.

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Chapter 1

SALMONELLA INTERNALIZATION FROM CONTAMINATED SEEDS OR IRRIGATION WATER IN GREENHOUSE TOMATOES

Introduction

The consumption of fruits and vegetables has been increasing throughout recent years. This is a likely consequence of a more health conscious society as well as research that correlates the increased consumption of fresh fruits and vegetables with decreased risk of stroke, high blood pressure, and cardiovascular disease *(27)*. There has also been an increase in foodborne illnesses related to fruits and vegetables. It is estimated that in the 1970s produce related outbreaks averaged two per year, and in the 1990s this number had risen to an average of sixteen per year *(45)*. The United States is one of the world's largest tomato producers, and approximately 5 billion pounds are consumed in the country each year *(11)*; considering the large-scale production and consumption of tomatoes, an outbreak has the potential to be particularly devastating. Over the last two decades an increased number of foodborne outbreaks have been associated with tomatoes, a large portion of these caused by *Salmonella (13, 24, 46)*.

Salmonella is one of the more prevalent foodborne pathogens in the United States and is responsible for an estimated 1.5 million cases of infection, 15,000 hospitalizations, and 500 deaths annually in the United States *(22, 35)*. Symptoms of *Salmonella* include

fever, diarrhea, vomiting, abdominal cramping, and nausea, and typically only last one to two days but is host dependent *(30)*. Although, the infective dose of *Salmonella* is unknown, the infective dose may be as low as 15 to 20 cells *(15)*. *Salmonella* has generally been associated with animal based products such as poultry, beef, eggs, and milk but has recently been linked to fresh produce including cantaloupes *(9, 10)*, lettuce and bagged salads *(25, 42, 47)*, mangoes *(38, 44)*, seed sprouts *(19, 39)* and tomatoes *(11, 24, 46)*.

A previous study has tested *Salmonella'*s ability to contaminate fruit from inoculated blossoms and inoculated stems and found that 37% and 40% of the fruit respectively was positive for *Salmonella (21)*. Guo et al.*(23)* also tested and found *Salmonella* to be present in hypocotyls, cotyledons, and stems of hydroponics tomatoes grown in the presence of contaminated nutrient solution. Ibarra-Sanchez et al. *(27)* tested the internalization of *Salmonella* that had been inoculated by dipping or spraying the tomatoes and then treated with rinsing in tap water, hypochlorite solution, or lactic acid solution Both the lactic acid and chlorine treatments showed a significantly decreased surfaces population that was below detectible limits *(27)*.

The objective of this study was to determine the internalization of *Salmonella* to greenhouse tomato plants and fruit from contaminated irrigation water. Tomato plants were treated over a period of one to 84 days with a suspension of *Salmonella* Montevideo inoculated water. This study also determined the survival of *Salmonella* in three different fertilizer stock solutions and the diluted (1.6%) fertilizer irrigation water. *Salmonella* levels were monitored in the six fertilizer solutions and a control over 24 hour intervals for a total of 72 hours.

Materials and Methods

Bacteria Culture and Inoculum Preparation. A strain of nalidixic acid resistant *Salmonella* Montevideo originally isolated from a tomato related outbreak was utilized in this study. The strain was grown in tryptic soy both (TSB) and transferred three times at 24 hour intervals. The last transfer culture was then plated on tryptic soy agar (TSA) and a single colony was selected to inoculated 9 mls of TSB. *Salmonella* spp. was confirmed using a 20E API strip (bioMerieux, Hazelwood, MO). Each TSA suspension was grown for 24 hr at 37°C. After 24 hr the suspension was centrifuged for 20 minutes at 7,500 X g and the spent medium was decanted. The cultures were then re-suspended in sterile distilled water to create a 10^8 cfu/ml suspension. Additional research was completed to demonstrate that the distilled water transporting medium had no effect on the survival of the *Salmonella* (See Appendix I)

***Salmonella* Survival in Commercial Fertilizers**. Six fertilizer samples and a control were inoculated with *Salmonella* (to demonstrate its survival). All fertilizer samples were collected from the Virginia Tech greenhouses; sample CF-S (Electrical Conductivity (EC)- 70.61 mS/cm, pH-4.41) was a commercial 20N-4.4P-16.6K fertilizer (Scotts Co., Marysville, OH) stock solution; Fertilizer CF-1.6 (EC-1.59 mS/cm, pH-6.66) is the diluted solution of the commercial 20N-4.4P-16.6K fertilizer that has been through a fertilizer injector (Docetron International, Clearwater, FL.) to create a 1.6% fertilizer water solution. Fertilizer US-S (EC- 38.30, pH- 7.82) is a stock solution of 11.3 kg UltraSol (SQM Corp, Atlanta, Georgia), 4.5 kg Epsom Salts, and 2.3 kg 0N-0P-43.2K

in 113.7 L water; fertilizer US-1.6 (EC-0.60, pH-7.96)is the 1.6% dilution of fertilizer US. Fertilizer CN-S (EC- 99.20, pH-5.70) is a stock solution of 11.3 kg Calcium Nitrate and 56.7 g Iron chelate (10%) to 113.7 L water; fertilizer CN-1.6 (EC- 2.61, pH- 6.53) is the 1.6% dilution of fertilizer CN-S. The seventh sample is a control consisting of sterile distilled water. Each sample was inoculated with 1 ml of 10^8 *Salmonella* suspension as prepared above. The samples were then spread plated on Hektoen Enteric in serial dilutions at 0, 24, 48 and 72 hr monitoring. Sample A was also enriched in Selenite Cysteine for 24 hr at 37°C and streaked on HE agar at 24, 48 and 72 hr to quantify the presence of *Salmonella*.

Tomato plants. Cultivar Trust tomato seeds were purchased from Coor Farm Supply (Smithfield, NC), and grown to seedlings in 46 cm^3 plug trays containing a commercial planting medium. Each plug tray contained 54 uninoculated seeds and 15 *Salmonella* inoculated seeds; plugs containing inoculated seeds were separated and placed in a plastic saucer to collect any contaminated runoff water. Seedlings were grown in plug trays for 40 days to the four true leaf stage. Thirty five seedlings from uninoculated seeds and ten from inoculated seeds were selected for uniformity and transplanted to 30.48 x 27.94 x 22.86 cm Dutch Bato Buckets (Coor Farm Supply, Smithfield, NC) containing aged loblolly pine bark medium mixed with 833 g/m^3 micronutrients (Micromax, Scotts Co, Marysville OH.) and 3.3 kg/m^3 lime. Tomato buckets were placed in 40.64cm plastic saucers to collect any water runoff. Three repetitions of 45 plants each were completed; two repetitions of plants were grown in university greenhouses on the Virginia Tech campus that was kept at a mean temperature of 21°C; the third repetition was grown in the university owned Clover Hollow

greenhouse in Newport Virginia (kept at a mean temp of 19°C). Both greenhouses were comparable in structure and locations were comparable in environmental conditions. Greenhouses contained ridge vents for cooling, and evaporative cooler and a gas heater. Fertilizer was donated by Coor Farm Supply, and mixed in two batches of stock solution. The first batch consisted of 11.3 kg UltraSol (SQM Corp, Atlanta, Georgia), 4.53 kg Epsom Salts, and 2.27 kg 0N-0P-43.2K fertilizer to 113.7 liters water; the second batch consisted of 11.3 kg Calcium Nitrate and 56.7 grams of Iron Chelate to 113.7 liters of water. Plants were fertilized daily and alternating each fertilizer every other day.

Inoculum Procedure. Eight different groups of plants were tested depending on inoculation technique and watering schedule (Table 1). Groups 1 through 6 each consisted of five plants each and were inoculated through watering seedlings with *Salmonella* contaminated water. Group 7 consisted of five uninoculated plants held as a control, and Group 8 consisted of 10 plants inoculated through seeds. Groups 1-6 were inoculated according to the schedule below by watering with 350 ml of 10^7 *Salmonella* suspension. Each 350 ml bottle was taken from a batch of 1800 ml 10^7 suspension was prepared for each group of plants. Eighteen ml of TSB were inoculated with *Salmonella* and then incubated for 24 hr at 37°C to grow the culture. The eighteen ml tubes were then centrifuged at 7,500 X g for 20 minutes. The pellet was re-suspended in 18 ml of sterile 0.1% peptone water. This suspension was added to 1782 ml of sterile distilled water to create a 10^7 suspension. Each batch of 1800 ml suspension was measured into individual sterile 500 ml bottles in 350 ml portions to water a single plant from the respective group. Group 8 plants were seed inoculated by soaking seeds on sterile blotter paper in a solution containing 10^8 *Salmonella* for 24 hr at room temperature.

Tomato Selection and Microbial Detection. Tomatoes were harvested from plants when they were 'red ripe'. Each tomato was placed in a sterile whirlpack bag. The plant the tomato was sampled from and fruit weight were recorded. Tomatoes were surface sanitized by dipping in a 70% ethanol solution and then allowed to dry under a flow hood. The tomatoes were then placed in a sterile stomacher bag containing 0.1% peptone water and hand rubbed for two minutes, as described by Guo *(21)*. The wash water was streaked onto HE agar to test for any bacteria not killed by the ethanol wash. The tomatoes were dissected using a sterile scalpel into stem scar and pulp. Each sample was placed in a sterile stomacher bag with 9 ml of sterile 0.1% peptone water and stomached for 2 minutes. One milliliter of sample was added to 9 ml of Selenite Cysteine broth and incubated for 24 hours at 37°C. Selenite Cysteine enrichment was then streaked onto HE agar that was incubated for a further 24 hours at 37°. *Salmonella* were identified by typical colony formation.

Tomato Plant Tissue Sampling. One tomato plant from each group was sampled and tissues from roots, stems, and leaves were sampled. The stems and leaves were both surface disinfected by spraying with a 70% ethanol solution and allowed to dry under a flow hood. After surface disinfection the stems were sampled in 3 cm portions beginning at 20, 40, and 60 cm. The roots were washed in tap water and dipped in a 1% sodium hypochlorite solution for two min, re-rinsed with sterile tap water, disinfected with a 70% ethanol spray and allowed to dry under a hood. Each of the tissue samples were combined with 10 ml of 1 M $MgSO_4$ and stomached for two min, 1 ml of the homogenate was used to inoculate 10 ml of selenite cysteine broth. This broth was

incubated at 37° C for 24 hr and streaked onto HE agar that was incubated for an additional 24 hr at 37° C. Presumptive *Salmonella* colonies were identified by typical colony formation with H_2S production and confirmed with a 20E API test (bioMerieux, Hazelwood, MO).

Statistics. All experiments were performed in three replications, and *Salmonella* counts were reported in their $\log_{10}$ values. Fertilizer samples were analyzed by Tukey's mean comparisons at P=0.05. Statistics were done using Statistical Analysis Software (SAS Institute Inc, Cary, NC).

Results and Discussion

Out of 128 tomatoes tested none were positive for the presence of *Salmonella* in either the stem scar or the fruit pulp. Of the tissue samples taken, no stem or leaf samples were positive for *Salmonella*. Five root samples were positive for *Salmonella*; three of these samples came from the group six plants, and two others from group four and five of the third rep. Any plants grown from contaminated seeds also did not yield any fruit or plant tissues positive for *Salmonella*.

There is little evidence that *Salmonella* is able to enter tomato plant systems through contaminated irrigation water. Jablasone et al. *(29)* tested the internalization of *Salmonella* in tomato fruit and tissues using bioluminescence, and found no evidence that *Salmonella* is able to contaminate tomato fruit and tissues through contaminated water in soil *(29)*. In conjunction with the results from this study it would suggest that *Salmonella* is not able to be transported through the plant tissue to the fruit. However, there has been evidence to suggest that *Salmonella* is able to internalize in other produce such as bean sprouts. Warringer et al. *(52)* demonstrated the ability of both *Salmonella* Montevideo and *E. coli* O157:H7 to internalize in bean sprouts that had been dipped for 20 minutes in a contaminated suspension. There is also evidence to suggest that *Salmonella* is able to internalize in tomato plants that have been hydroponically grown with a contaminated nutrient solution. Guo et al. *(23)* found *Salmonella* to be present in hypocotyls and cotyledons within one day of exposure to contaminated nutrient solution containing 4.46 to 4.65 log CFU/ml *Salmonella*.

Salmonella is able to enter a fruit though natural damage such as cuts and punctures. There is also evidence that bacterial pathogens such as *Salmonella* may be able to enter fruit through cuts or its stem scar when place in contaminated water; fruit is particularly susceptible if the water is held at a cooler temperature than the fruit *(17)*. This is important to post-harvest processes that may further spread contamination, such as water baths the fruit may be placed in. Packinghouses must use proper controls to ensure that water baths are free from contamination and held at proper temperatures.

Salmonella was found in the roots of plants from group 6 of all three repetitions. Group 6 received the greatest number of *Salmonella* treatments, as well group 6 was also the last to be treated so there was less time between group 6 treatments and sampling. The third repetition of this study was grown in a greenhouse in Newport Virginia, whereas the first and second repetitions were grown in the same university greenhouse in Blacksburg, Virginia. The third repetition also had *Salmonella* positive root samples from groups 4 and 5. These samples had also received the treatment closer to the sampling time than the other two repetitions because the repetitions were staggered, and that may explain the *Salmonella* surviving. A separate study found that an approximate value of 1 log cfu/ml may be filtered from the initial watering dose and into the pine bark medium (Appendix II).

There are several reasons that may explain the *Salmonella* contamination in the roots. Roots may have become injured through natural growth and provide an entry point for the bacteria. The staking process may have also led to unavoidable root damage. There is also a possibility for error if *Salmonella* evaded the root sanitizing process by adhering to small soil particles.

The fertilizer 1.6% diluted solutions (CF-1.6, US-1.6, and CN-1.6) yielded results that were not significantly different from each other, the control or stock solution US-S (Table 2); there was not a significant decrease of *Salmonella* levels in any of these solutions. CN-S was not proven to be significantly different to only fertilizer US-S. CF-S was significantly different from all other treatments, and after 24 hours was no longer enumerable.

Many farms use concentrated fertilizer solutions that can be diluted and pumped into irrigation water. A device such as a docetron combines the concentrated fertilizer stock solutions with water to create a desired dilution. The dilution value of fertilizer applied to the tomatoes in this experiment was 1.6%, and that was therefore the dilution used for the *Salmonella* survival in fertilizer study. The study results showed that all the diluted samples allowed for *Salmonella* survival, and were not significantly different than the control. Although the *Salmonella* were able to survive in the diluted fertilizer solutions, this may not pose a problem to farms because practices recommend that the fertilizer and irrigations lines be flushed after use *(31)*. However, fertilizer stock solutions are mixed in large batches that may be held for a period of time. The batches may become contaminated through previously contaminated water or an outside source such as birds and reptiles. In case of contamination of the stock solutions, bacteria would not be able to survive in the CF-S solution after 24 hours, and in CN-S would have a 4.48 log difference over 72 hours (Figure 1).

The results of this study suggests that tomato fruit and tissues above the root system cannot be contaminated by *Salmonella* after biweekly treatments of contaminated water to greenhouse tomato plants contained in a pine back medium. Further research

needs to be done to examine the internalization of *Salmonella* in tissues and tomato fruit grown in field conditions. The results of this experiment would also suggest that further research should be done to explore the transmission of *Salmonella* internalization into tomato fruit through common post harvest practices.

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Table 1. Watering schedule of *Salmonella* treatments to tomato plants where day 1 is the first day after seedlings were transplanted from plug trays. On inoculum watering days each plant in the group(s) treated received 350 ml of 10^7 cfu/ml Salmonella in deionized water suspension.

Experimental Group	Day 1	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
1	O	X	X	X	X	X	X
2	O	O	X	X	X	X	X
3	O	O	O	X	X	X	X
4	O	O	O	O	X	X	X
5	O	O	O	O	O	X	X
6	O	O	O	O	O	O	X
7 (control)	X	X	X	X	X	X	X
8 (seed inoculated)	X	X	X	X	X	X	X

X – regular watering, O – inoculum watering

Table 2: Log_{10} counts (cfu/ml ± SD) of *Salmonella* in 6 different fertilizer solutions and one control over a 72 hour period when held at room temperature.

	Time			
	0 Hour	24 Hour	48 Hour	72 Hour
CF-S	6.41± 0.37	0 ± 0.0	0 ± 0.0	0 ± 0.0
CF-1.6	6.89 ± 0.31	7.56 ± 0.69	6.74 ± 0.12	6.66 ± 0.11
US-S	6.86 ± 0.10	5.57 ± 0.63	4.54 ± 0.21	4.35 ± 0.73
US-1.6	6.86 ± 0.14	7.13 ± 0.44	6.59 ± 1.02	7.02 ± 0.70
CN-S	6.74 ± 0.08	6.21 ± 1.01	2.46 ± 0.94	2.26 ± 0.30
CN-1.6	6.71 ± 0.03	7.27 ± 0.43	7.33 ± 0.80	6.53 ± 0.66
Con	6.93 ± 0.28	6.93 ± 0.31	6.66 ± 0.38	6.90 ± 0.45

CF-S commercial 20N-4.4P-16.6K fertilizer stock solution; CF-1.6 is the 1.6% fertilizer diluted solution of the commercial 20N-4.4P-16.6K fertilizer. US-S is a stock solution of 11.3 kg UltraSol , 4.5 kg Epsom Salts, and 2.3 kg 0N-0P-43.2K in 113.7 L water; US-1.6 is the 1.6% dilution of fertilizer US. Fertilizer CN-S is a stock solution of 11.3 kg Calcium Nitrate and 56.7 g Iron chelate (10%) in 113.7 L water; CN-1.6 is the 1.6% dilution of fertilizer CN-S. The seventh sample is a control consisting of sterile distilled water

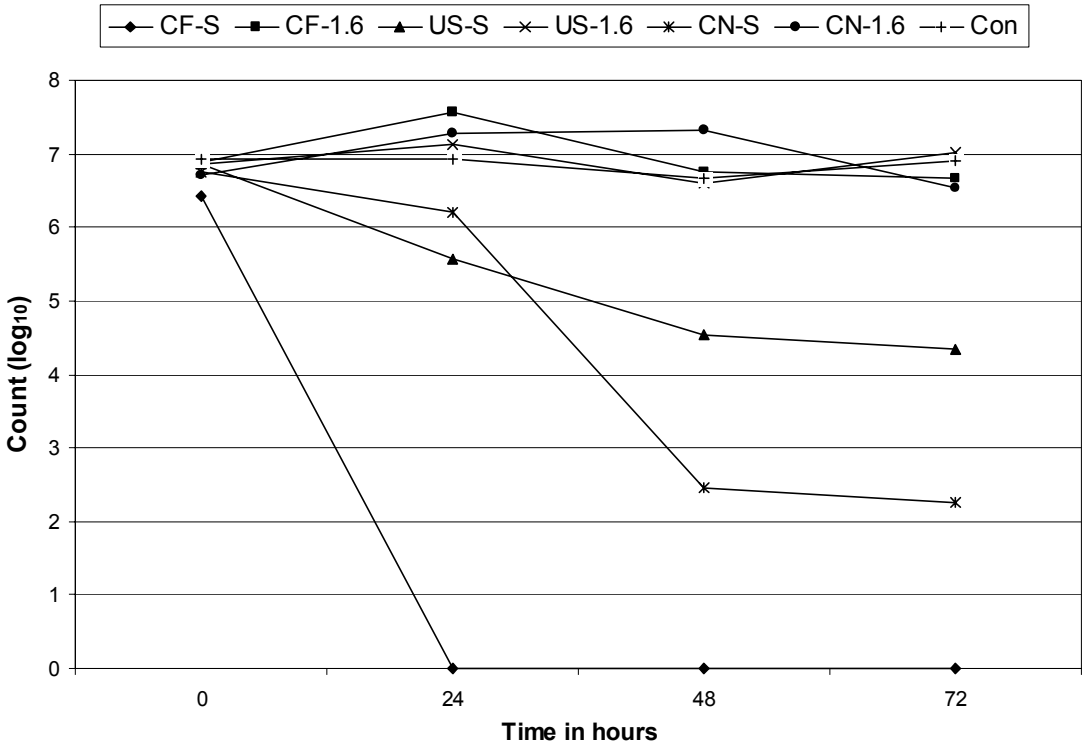
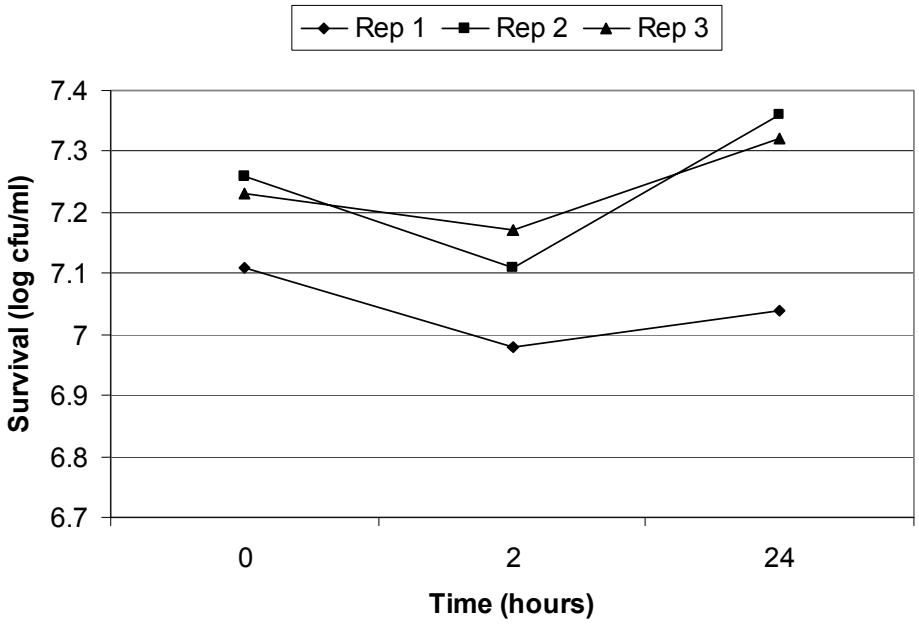


Fig 1: Log_{10} (cfu/ml) counts (n=3) of *Salmonella* in three different fertilizer solutions, fertilizer 1.6% dilutions, and a sterile distilled water control over 72 hours

APPENDIX I

Salmonella Survival in Sterile Distilled Water Over a 72 Hour Period

Methods: 2.5 ml of 10^8 cfu/ml *Salmonella* suspension was added to 247.5 ml of sterile distilled water to create 250 ml of 10^6 cfu/ml *Salmonella* suspension. Samples were collected at 0, 2, and 24 hours and plated in serial dilutions on Hektoen enteric agar. Plates were incubated for 48 hours at 37°C and counted.



Log_{10} counts (cfu/ml) *Salmonella* Survival in Sterile Distilled Water over 24 Hours

APPENDIX II

Salmonella Filtration through Aged Loblolly Pine Bark

Methods: 2 ml of approximately 10^8 cfu/ml *Salmonella* in DI water suspension was added to 198 ml of sterile deionized water to create a 10^6 cfu/ml *Salmonella* suspension (initial level reported below). The 200 ml suspension was watered on 700 grams of aged loblolly pine bark and allowed to drain for 30 minutes. The runoff water was collected and plated on Hektoen Enteric agar that was incubated for 48 hour at 37°C and counted.

Table 3. Demonstration of *Salmonella* absorption in aged loblolly pine bark across three repetitions.

Replication	Initial Inoculum Level	Runoff Level
1	6.76	5.44
2	6.65	5.82
3	7.07	5.43