Detection and monitoring of microbes of concern in animal production environment

by

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Abstract

An estimated 48 million Americans become ill, 128,000 are hospitalized, and 3,000 die of foodborne illnesses each year, and foodborne illness costs the United States $14.6-16.3 billion annually. Because foodborne illness continues to be costly, in terms of money and health, in the United States, new aspects of food protection are being evaluated including microbial contamination of the crop production environment.

This study evaluated two separate aspects of environmental microbial contamination. The first portion of this study evaluated the presence and spread of *Salmonella* spp. in the environment surrounding the Auburn University Poultry Research Unit. Two Nalidixic acid resistant *Salmonella* strains were used for tracking the spreading of *Salmonella* spp. throughout the environment. Nalidixic acid resistant strains were isolated from environmental samples 0.44 km and 0.38 km away from the central location of the poultry farm. This signifies that environmental carriers/factors could contribute to the spreading of *Salmonella* spp. In addition, *Salmonella* serogroup B was isolated from 8 out 13 sampling locations throughout a 3-month time span. Serotyping of these isolates showed that they are *Salmonella* Saintpaul. Pulse Field Gel Electrophoresis (PFGE) was performed and showed that those isolates are genetically identical.

The second portion of this study focused on the application of microbial water safety guidelines set forth by the 2016 Food Safety Modernization Act (FSMA): Produce Safety Rule. Six farms in the East Central Alabama area were selected, and water samples were collected once a month from October 13, 2015 to June 20, 2016 from five

farms with ponds on them. Four out of the six farms have streams running through them. For these, surface water samples were collected at the site where the stream enters and exits the farm. Each point that was sampled was considered a site, and there were a total of thirteen sites. Following the U.S. Environmental Protection Agency (USEPA) method 1603, generic *Escherichia coli* were enumerated using modified mTEC agar. Geometric Mean (GM) and Statistical Threshold Value (STV) were calculated using Cornell University's Produce Safety Alliance Excel tool. Although differences were seen in *E. coli* numbers from samples collected in different months, based on the calculations, water quality of five sites is in compliance with water quality microbial requirements. Eight sites had GM and/or STV values that did not comply with these standards. However, by using the Excel tool, a die-off period of one day is suggested for seven of them, and a two-day die-off period is suggested for one.

For all four streams sampled, the outgoing stream sites did not consistently show higher generic *E. coli* concentrations than the incoming stream sites. No conclusion can be made at this point about whether animal agriculture has significant impact on microbial surface water quality. However, land use, geographical layout, weather variations, and even surrounding land factors can affect the microbial quality of surface water sources (Mallin, Johnson, & Ensign, 2008). For these reasons, it is important that each water source to be used for irrigation be monitored to prevent crop contamination.

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Chapter 1: Literature Review

1.1 Microbial Contamination in the Environment

The gastrointestinal tract of livestock, fowl, domesticated, and wild animals is considered to be the primary habitat of *Escherichia coli* and *Salmonella* spp. The secondary habitat of these microorganisms includes water, sediment, and soil (Savageau, 1983). Pathogens in the gastrointestinal tracts of livestock and other animals are deposited into the environment through fecal excrement. Runoff water from the land washes fecal material and associated microbes into surface and groundwater sources. Water from these sources may be used on the farm for irrigation, produce washes, or cleaning. Use of contaminated water may lead to the contamination of produce or food contact surfaces, which may contribute to the spread of pathogenic bacteria (Figure 1). The secondary habitat, and the microbes' ability to persist and survive within them pose a major concern to public health. It is through this secondary habitat of soil, water, and sediment that pathogens can be spread during production, harvest, processing, or packaging and can contaminate meat, produce, and other food commodities. Vectors such as humans, flies, wild birds, and farm equipment can carry and transfer pathogens from the environment to food and food contact surfaces (Winfield & Groisman, 2003).

1.1.1 Microbial Contamination of Surface Water by Agricultural Runoff

Water pollution caused by animal agriculture is typically defined as nonpoint source (NPS) pollution. Agricultural activities that can have major negative impacts on water quality include but are not limited to poorly located or managed animal feeding operations, manure spreading, overgrazing, plowing too often or at the wrong time, and

improper application of fertilizers (EPA, 2016). The United States Environmental Protection Agency (USEPA) (2016) reports, "Agricultural NPS pollution is the leading source of water quality impacts on surveyed rivers and streams, the third largest source for lakes, the second largest source of impairments to wetlands, and a major contributor to contamination of surveyed estuaries and ground water." Fecal excrement from grazing livestock is typically deposited into surface water sources by runoff from the land (Jamieson, Gordon, Joy, & Lee, 2004). Stormwater runoff has been shown to have a large impact on surface water quality regardless of land use. Positive correlations have been shown between rainfall and total suspended solids (TSS), turbidity, fecal coliform abundance, phosphorus, and biochemical oxygen demand (BOD) in streams of urban, suburban, and rural areas. A subsequent increase in TSS and fecal coliform abundance have been linked to the association of microorganisms with soil particles. Once these particles and microorganisms are deposited into the water, they can remain suspended or deposit into the sediment (Mallin, Johnson, & Ensign, 2008). Once deposited into the sediment, bacteria such as fecal coliforms and *E. coli* persist and serve as significant sources of waterborne microbial contaminants (Garzio-Hadzick, Shelton, Hill, Pachepsk, Guber, & Rowland, 2010).

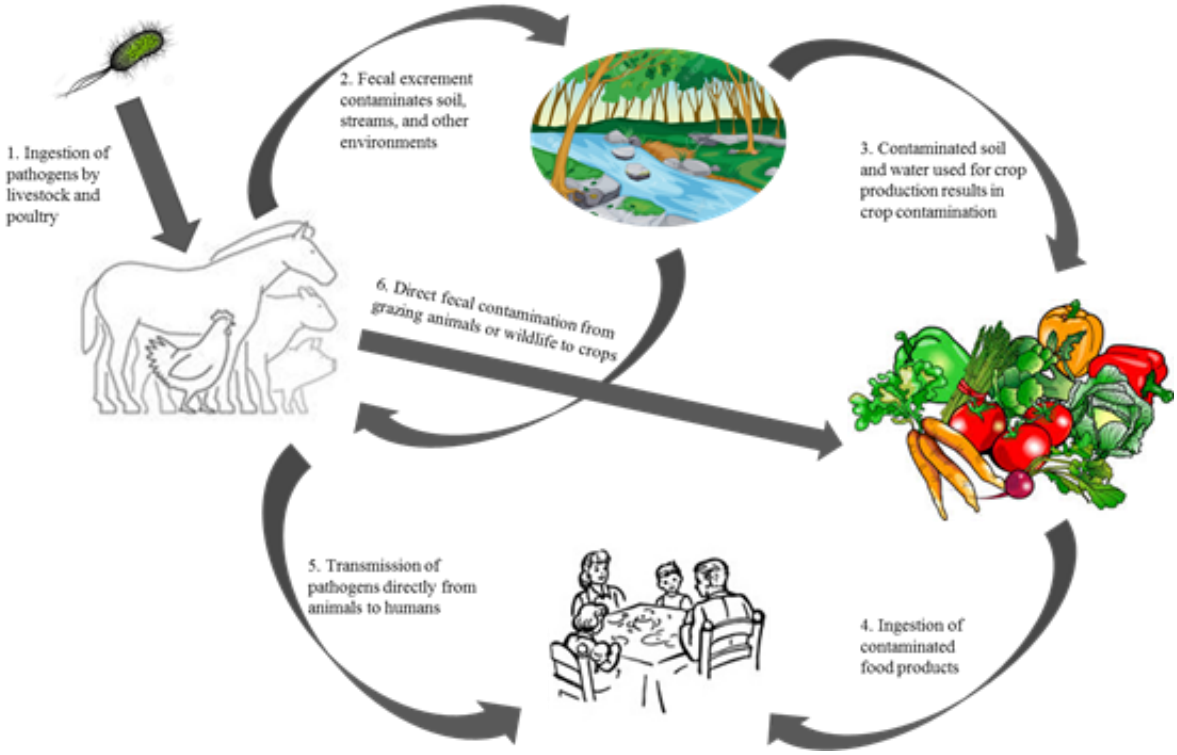

Figure 1: Illustration of contamination pathways leading to foodborne illness.

1.2 Microbial Contamination of Food

1.2.1 Microbial Contamination of Meat and Meat Products

Because *Salmonella* spp. and *E. coli* are common enteric microorganisms, it is possible that meat and meat products may become contaminated during the harvesting of the animal. In addition to the gastrointestinal tract, Swanenburg, Urlings, Snijders, Keuzenkamp, and van Knapen (2001) isolated *Salmonella* spp. from the tonsils, livers, tongues, and mesenterial lymph nodes of swine carcasses. In the same study, *Salmonella* spp. were isolated from environmental areas in the slaughter houses including drain water and processing equipment such as the carcass splitter. Knowing that potential pathogens such as *Salmonella* spp. can be found in various parts of animal carcasses and inside

processing facilities allows for the development of improved harvesting, cleaning, sanitizing, and preventative measures.

Meat and meat products can also become contaminated with further handling and processing. Pathogens and other microbes may be introduced into processed meat products and ready-to-eat meat products through contact with contaminated food contact surfaces, packaging materials, poor hygiene practices of employees, or the incorporation of contaminated additives. Maintaining a safe food supply means identifying the sources of contamination and implementing preventative measures to eliminate hazards at each point.

1.2.2 Microbial Contamination of Produce

Contamination of fresh produce can occur virtually anywhere throughout the production process. To minimize foodborne outbreaks associated with fresh produce, it is important to identify the points at which the crops become contaminated. Contamination may occur preharvest, during harvest or processing, or at the consumer level. Several practices have been identified as points of crop contamination. Contaminated soil, irrigation water, farm equipment, workers, and processing equipment have been identified as potential vectors of contamination (Brackett, 1998).

Preharvest contamination may occur if crops are exposed to contaminated soil, irrigation water, wildlife, or manure spread as fertilizer (Hanning, Nutt, & Ricke, 2009). The use of manure and irrigation water contaminated with *E. coli* O157:H7 has been shown to increase the presence of these microorganisms on and in vegetables such as lettuce (Solomon, Yaron, & Matthews, 2002). Many pathogens such as *Salmonella* spp.

and *E. coli* are carried by grazing livestock, and the microbial water quality is greatly affected by runoff from land grazed by these animals (Hubbard, Newton, & Hill, 2004). The surfaces of crops may become contaminated by the use of surface water that has been negatively impacted by runoff (Hanning et al., 2009). This can result in the colonization of crops with biofilms or the internalization of microbes by the plants (Erickson, 2012).

Additionally, crops may become contaminated with feces through contact with wildlife. The CDC (2015a, 2015b) states that *Salmonella* and *E. coli* are widely dispersed in nature and are commonly found in the intestinal tracts of livestock, wildlife, and domestic pets. Contact with fecal material from these animals can potentially result in the contamination of crops.

1.3 Pathogens of Concern

1.3.1 *Salmonella* spp. and Salmonellosis

The FDA Bad Bug Book describes *Salmonella* as a motile, non-spore forming, Gram negative, rod-shaped bacterium belonging to the family, *Enterobacteriaceae*. The genus *Salmonella* is divided into two species that can cause illness in humans: *S. enterica* and *S. bongori*. *Salmonella enterica*, which is of the greatest public health concern, is comprised of six subspecies and further divided into over 2,500 serotypes based on the Kaufmann-White typing scheme. *Salmonella* spp. can cause two types of illness: nontyphoidal salmonellosis and typhoid fever. Nontyphoidal salmonellosis is caused by *Salmonella* serotypes other than *S.* Typhi and *S.* Paratyphi A (FDA Bad Bug Book, 2012) and will be the main focus of this study.

The infectious dose of nontyphoidal *Salmonella* spp. greatly depends on the strain that is ingested and on the susceptibility of the host. Host adaptation and the presence of virulence genes account for differences in virulence among different *Salmonella* spp. serotypes. Host factors that affect the infectious dose and severity of illness include but are not limited to age, recent use of antibiotics, immune status, and gastric acid production (Blaser & Newman, 1982). While there are numerous factors that can affect the infectious dose (Blaser & Newman), the FDA Bad Bug Book reports that the infectious dose of *Salmonella* spp. can be as low as 1 cell in the very young, very old, or immunocompromised individuals (2012).

According to the FDA Bad Bug Book (2012), symptoms of Salmonellosis typically include nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. Septicemia or bacteremia may occur in some infections when *Salmonella* cells escape the gastrointestinal tract and infect the blood or organs of patients. Symptoms usually last between four to seven days depending on the host, the dose ingested, and the strain ingested. One complication that has been reported in 2% of culture proven cases is reactive arthritis, which occurs as an inflammatory immune response to the infection. This response may last for months or years and can lead to chronic arthritis, irritation of the eyes, and difficulty urinating (CDC, 2015b).

For most serotypes of Salmonella, the mortality rate is less than 1%, however, some circumstances may lead to a higher mortality rate. For example, *S.* Choleraesuis may have a mortality rate as high as 20%, S. Dublin has a 15% mortality rate in elderly patients due to septicemia, and *S.* Enteritidis has a mortality rate of 3.6% in nursing homes and hospitals (Iowa State, 2005).

Salmonella spp. are commonly found in the intestinal tracts of many farm animals, humans, birds, and reptiles and are dispersed throughout nature (FDA Bad Bug Book, 2012). It is through fecal excrement that these bacteria contaminate the soil and water (Andino & Hanning, 2014). Baudart, Grabuolos, Barusseau, and Lebaron (2000) compared fecal indicators and *Salmonella* spp. discharges from the Mediterranean coastal watershed, a common characteristic of cattle, poultry, and swine rearing. This study demonstrated an increase in fecal coliforms and *Salmonella* which positively correlated with storm events. This was associated with the flow of sediments and associated microbes being washed into the water. In the absence of storm activity, microbial concentrations were also higher in the sediments than suspended water. Because disturbance events can lead to the resuspension of sediment and microbes, the microbes associated with sediment particles serve as an important source of contamination (Baudart et al., 2000).

Meat, irrigation water, produce, equipment, workers, and utensils may be contaminated with *Salmonella* spp. and may contribute to the spread of salmonellosis infections (FDA Bad Bug Book). Since rivers, lakes, and wastewater systems are commonly used as irrigation water for crops (Nutt, Pillai, Woodward, Sternes, Zabala-Diza, Kwon & Ricke, 2003), it is important that water contamination be considered a food safety concern.

1.3.2 *Salmonella* spp. Outbreaks

In the past, *Salmonella* spp. were typically thought of as being associated with animal products; however, *Salmonella* spp. outbreaks have recently been associated with

fresh produce (FDA Bad Bug Book, 2012). So far in 2016, the CDC has reported three foodborne *Salmonella* spp. outbreaks in the United States. *Salmonella* Montevideo and *Salmonella* Senftenberg outbreaks linked to Wonderful Pistachios have resulted in eleven illnesses and two hospitalizations across nine states. Contaminated alfalfa sprouts were the source of a *Salmonella* Muenchen and *Salmonella* Kentucky outbreak that affected twenty-six people and hospitalized eight across twelve states. RAW Meal Organic Shake & Meal products were linked to a *Salmonella* Virchow outbreak that affected thirty-three people and led to six hospitalizations across twenty-three states (CDC, 2016a).

1.3.3 Pathogenic *Escherichia coli*

Escherichia coli are Gram-negative, rod-shaped, non-spore forming, facultative anaerobic bacteria belonging to the Enterobacteriaceae family (Singleton, 1999). *E. coli* are a large, diverse group of bacteria found in the environment, in foods, and in the intestines of people and animals. Many strains of *E. coli* are a harmless part of the normal gut flora, but others can cause a wide variety of illnesses such as diarrhea, urinary tract infections, respiratory illness, pneumonia, and other illnesses (CDC, 2015a). While most strains of *E. coli* are harmless, six pathogenic groups have been identified based on their virulence properties. These six groups are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Pathogenic *E. coli* are grouped based on their virulence properties (FDA Bad Bug Book, 2012).

Enterotoxigenic *E. coli* possess the ability to produce heat-labile (LT) toxin and heat-stable (ST) toxin. ETEC also possess several colonization-factor antigens. ETC typically causes gastroenteritis in humans and is associated with travelers' diarrhea. The infective dose is high, ranging from 10 million to 10 billion cells in adults, but may be lower in children. ETEC causes non-bloody, watery diarrhea which can lead to dehydration. Patients who are severely affected or have a weakened immune system may require hospitalization. While ETEC is not commonly reported in the United States, it is reported to cause 380,000 deaths worldwide each year. Humans are the main carrier of ETEC, and contaminated food is typically associated with the use of sewage-contaminated water or contamination by food handlers (FDA Bad Bug Book, 2012).

Enteropathogenic *E. coli* (EPEC) are characterized by the presence of the locus for enterocyte effacement (LEE) pathogenicity island. This locus can carry many virulence factors including the gene that encodes for intimin and intimin receptors. These two genes allow the EPEC to adhere to intestinal epithelial cells. EPEC is often responsible for infantile diarrhea in the United States. Mortality rates can range from 25% to 50% depending on the availability of medical treatment. The infective dose is assumed to be very low in infants and much higher in adults. Foodborne outbreaks of EPEC are rare and the sources are not fully understood (FDA Bad Bug Book, 2012).

Enterohemorrhagic *E. coli* (EHEC) are characterized by their production of Shiga toxins. There are many serotypes of ETEC, but the best classified is *E. coli* O157:H7. O157:H7 accounts for 75% of EHEC infections. Other EHEC are collectively referred to as non-O157 strains. EHEC can have a wide range of symptoms but typically causes hemorrhagic colitis, and abdominal cramps. The Shiga toxins can also cause kidney

failure, referred to as Hemolytic Urea Syndrome (HUS). M.A. Karmali reports that 3% to 7% of EHEC cases lead to HUS. HUS can cause acute kidney failure or permanent kidney damage, and is the leading cause of acute kidney failure in children (2004). According to the FDA Bad Bug Book, approximately 63,000 EHEC cases are reported in the United States, annually. Ground beef served as the first source of O157 infections and remains a major foodborne carrier. However, produce has become a major concern for EHEC outbreaks. The largest O157:H7 outbreak, in Japan, was associated with radish sprouts and affected 10,000 people. O157:H7 remains a concern as it has been implicated in outbreaks involving lettuce, salads, sprouts, spinach, and other produce and food products (FDA Bad Bug Book, 2012).

Enteroinvasive *E. coli* (EIEC) are characterized by their ability to invade colonic epithelial cells. EIEC does not have any reported deaths associated with its infections. Symptoms are typically very mild diarrhea and abdominal cramping. Humans are the only known reservoir for EIEC, and infection is usually the result of consumption of food or water contaminated with human feces (FDA Bad Bug Book, 2012).

1.3.4 Pathogenic *E. coli* Outbreaks

Currently, for the year of 2016, two foodborne *E. coli* outbreaks have been reported in the United States. One outbreak has been attributed to *E. coli* O121 (STEC O121) found in flour, which caused thirty-eight illnesses and ten hospitalizations across twenty states. The second outbreak has linked *E. coli O157* (STEC O157) to alfalfa sprouts produced by Jack & The Green Sprouts. This outbreak led to eleven illnesses and two hospitalizations over two states. In previous years, *E. coli* outbreaks have been

linked to chicken salad, Chipotle Mexican Grill, raw clover sprouts, ground beef, ready-to-eat salads, frozen food products, romaine lettuce, bologna, cheese, and many other sources (CDC, 2016b). *E. coli* has the potential to contaminate many different food sources and is a major foodborne pathogenic concern.

E. coli O157 is estimated to cause over 73,000 illnesses in the United States annually. Rangel, Sparling, Crowe, Griffin, and Swerdlow (2005) reviewed CDC outbreak reports from 1982-2002. In that period, 49 states reported 350 outbreaks, representing 8,598 cases, 1,493 (17%) hospitalizations, 354 (4%) hemolytic uremic syndrome cases, and 40 (0.5%) deaths. Foodborne transmission route accounted for 183 (52%) of these outbreaks, and waterborne transmission route accounted for 31 (9%) of these outbreaks. The food vehicle for 75 (41%) foodborne outbreaks was ground beef, and for 38 (21%) outbreaks, produce. Ground beef was the source of the first *E. coli* O157 outbreak in 1982 and remains the most common vehicle among foodborne outbreaks. Produce was first reported as an *E. coli* O157 vector in 1991 and has remained a prominent vector for *E. coli* transmission (Rangel et al., 2005).

1.4 Studies evaluating crop contamination and internalization of *Salmonella* spp. and pathogenic *E. coli*

Crops can become contaminated with pathogens by exposure to contaminated soil, water, machinery, workers, etc. In an effort to develop effective preventative measures, it is also important to discuss the mechanisms by which crops uptake these microbes. The methods of contamination seem to vary depending on the type of produce, pathogen characteristics, and source of contamination.

Lopez-Velasco, Sbodio, Tomas-Callejas, Wei, Tan, and Suslow (2012) used irrigation water contaminated with *Salmonella enterica* to evaluate the root uptake of *S. enterica* by melons during production. In this study, irrigation systems mimicked farming practices and used a high inoculum of *S. enterica.* They found that, under these conditions, the honeydew and cantaloupe plants were not able to uptake *S. enterica* from the contaminated irrigation water into their vasculature. However, an additional experiment in which the peduncle was injured and inoculated with contaminated water resulted in detectable *Salmonella* at the fruit abscission zone. It was also noted that the external surfaces of the melons, where soil contact occurred, were contaminated with the experimental *Salmonella*. The external surfaces of melons can be difficult to sanitize depending on the surface qualities, and this can lead to contamination of edible portions when cutting (Hanning et al., 2009).

E. coli O157:H7 has been found in the edible portions of lettuce after being grown in contaminated soil and irrigation water. Solomon et al. (2002) performed a study in which soil and irrigation water were inoculated with *E. coli* O157:H7 transformed with pGFP plasmid. *E. coli* O157:H7/pGFP was detected in the edible portions of the lettuce after harvesting. This is of great concern because the infective dose of *E. coli* O157:H7 can be very low and sanitation of the lettuce surface is ineffective in killing the internalized bacteria.

Zhuang, Beuchat, and Angulo (1995) demonstrated that *Salmonella montevideo* internalization into tomato tissues is influenced by differences in temperature of tomato storage and wash water. This study submerged 25°C tomatoes in 10°C, 25°C, and 37°C *S. montevideo* dip suspensions then stored them at 10°C or 20°C. Submerging tomatoes

in a 10°C suspension resulted in a significant internalization of *S. montevideo* cells. Subsequent storage of tomatoes at 20°C resulted in increases of *S. montevideo* populations over the course of the study. This study also evaluated the effectiveness of a two-minute chlorine disinfection dip at 60, 110, 210, and 320 ppm. Concentrations of at least 110 ppm free Cl^- were required for a significant reduction in *S. montevideo* at the stem scar, but the populations were not eliminated. The inability to completely eliminate the *Salmonella* populations at the stem scar is likely the result of internalization into the tomato tissues. The internalization of pathogens protects them from surface disinfection; thus, they remain viable for infection of the consumer.

Guo, Chen, Brackett, and Beuchat (2001) demonstrated that *Salmonella* spp. can become internalized by tomatoes using two different inoculation methods. In this experiment, five *Salmonella* spp. were used to inoculate tomato plants by stem injection before fruit set, by stem injection after fruit set, and by brushing flowers with inoculum. Stem injections were performed by depositing 50 µl of inoculum containing 7.5 $\log_{10}$ CFU onto the stem approximately 5 cm from the flower base followed by pricking with a 25-gauge needle. Flower inoculations were performed by soaking a small paintbrush in the same inoculum and gently brushing over the flowers. Negative control plants were grown under the same conditions but without treatment with *Salmonella* spp. The results showed that 43% of the tomatoes harvested from plants that received stem injections before fruit set tested positive for *Salmonella* spp. Forty percent of tomatoes harvested from plants that received stem injections after fruit set tested positive for *Salmonella* spp. Twenty-five percent of the tomatoes harvested from plants that received treatment by brushing flowers with inoculum tested positive for *Salmonella* spp. While the inoculum

level of *Salmonella* spp. is higher than what is typically encountered in nature, this study shows that injured stems or flower blooms may serve as possible routes of pathogen entry into tomatoes.

In a review by Hirneisen, Sarma, and Kniel (2012), several aspects were evaluated in the root uptake of pathogens by crops. While some literature seems to be contradictory, there is a lot of variation among experimental designs that contribute to differences in results. It can be concluded that production methods can have a large effect on the internalization of pathogenic bacteria into crops. Crops grown in inoculated hydroponic solutions seem to have higher incidence of pathogen uptake than those grown in inoculated soils, although studies have also shown that pathogen uptake can occur in crops grown in soil. Certain stressors seem to influence the root uptake of pathogens as well. Damage or injury to the root systems seems to provide access for some pathogens to enter the vascular system of the plants. It has also been shown that specific pathogens and pathogen strains can influence their ability to internalize in plant tissues. Hirneisen et al. also concluded that internalization through root systems is a plant-pathogen specific reaction, and that the internalization at root level does not mean pathogens will be found in the edible portions of the crops. "The variability in design and results likely better represents the realities and risks of internalization after a contamination event during produce production" (Hirneisen et al., p. 403).

1.5 The Importance of Produce Safety

Anekwe and Hoffmann (2013) reported two recent studies of foodborne illness cost to The U.S. Department of Agriculture (USDA). The first report, by the University

of Florida, estimates foodborne illness costs the United States $14.6 billion per year. The second report, by Ohio State University, estimates the cost of foodborne illness in the United States at $16.3 billion per year. Both reports are based upon foodborne illnesses, hospitalizations, and deaths reported by the Centers for Disease Control and Prevention (CDC). The two studies consider different medical complications associated with foodborne illnesses and use different methods to place value on lost pregnancies, deaths, and productivity. Nontyphoidal *Salmonella* accounts for approximately $3.4 billion each year, making it the costliest pathogen. *E. coli* O157:H7 accounts for approximately $300 million each year.

The CDC estimates that each year 48 million Americans get sick, 128,000 are hospitalized, and 3,000 die of foodborne illnesses. The CDC uses foodborne illness source attribution to estimate the number of illnesses associated with specific food sources (CDC, 2014). In effort to give an accurate estimation of foodborne illnesses in the United States, foodborne illness source attribution uses reported foodborne disease outbreaks and several statistical models to account for underreported and underdiagnosed illnesses (Scallan, Hoekstra, Angulo, Tauxe, Widdowson, Roy, Jones, & Griffin, 2011).

According to the data collected and analyzed for foodborne illness attribution from 2000 to 2008, it is estimated that 31 major pathogens acquired in the United States caused 9.4 million episodes of foodborne illness, 55,961 hospitalizations, and 1,351 deaths each year. Of the 55,961 hospitalizations, 64% were of bacterial cause, and non-typhoidal *Salmonella* spp. accounted for the most hospitalizations at 35%. *E. coli* O157 was estimated to cause the fifth most hospitalizations at 4%. Deaths related to foodborne illness of bacterial origin were estimated to account for 64% of the total. Off all

foodborne related deaths, non-typhoidal *Salmonella* spp. accounted for the most deaths at 28% (Scallan et al., 2011). It is important to understand the etiology of these pathogens in effort to develop and implement preventative strategies to protect the food system.

Determining the microbial agent associated with foodborne illnesses is just the first step in protecting the food supply. The next step is linking the microbial agent to the food vehicle which was contaminated. Using data reported to the Foodborne Disease Outbreak Surveillance System from 11998-2008, Painter, Hoekstra, Ayers, Tauxe, Braden, Angulo, and Griffin (2013) were able to estimate the attribution of foodborne illness, hospitalizations, and deaths to specific food commodities. For this analysis, food commodities were divided into three categories: aquatic animals, land animals, and plants. The plant category was further divided into three subcategories: grains-beans, oils-sugars, and produce. Produce was further divided into fruits-nuts and vegetables. Vegetables included fungi, leafy, root, sprout, and vine-stalk vegetables. It was estimated that 51% of foodborne illnesses were attributed to the plant commodities, with leafy vegetables accounting for 22% of the total illnesses. Forty-one percent of hospitalizations were caused by plant commodities, and leafy vegetables accounted for the second most hospitalizations with 14% of the total. Of the three categories, aquatic animal, land animal, and plant commodities, plant commodities accounted for 25% of deaths. Behind meat-poultry commodities, produce was identified as the second most lethal commodity accounting for 23% of the total deaths. Overall, the plant commodity group accounted for a greater proportion of illnesses linked to many bacterial sources including enterotoxigenic *Escherichia coli,* Shiga toxin-producing *E. coli* (STEC) O157, non-O157 STEC, *Salmonella enterica* serotypes Javiana, Newport, and others. It was

found that the plant commodity group accounted for the most bacterial related illnesses at 32%. Leafy vegetables accounted for more illnesses than any other commodity (22%), were the second leading cause of hospitalizations, and were the fifth most frequent cause of death. Poultry was associated with the most deaths (19%), and 26% of those deaths were attributed to *Salmonella* spp. (Painter et al., 2013). During an *E. coli* O157:H7 study done by Rangel et al. (2005), it was determined that produce was the second most commonly identified food vehicle for *E. coli* O157:H7, ranking behind ground beef. The Interagency Food Safety Analytics Collaboration (IFSAC) 2015 report noted that *Salmonella* spp. infections were widespread throughout many types of food commodities, whereas, *E. coli* O157 infections were mostly attributed to beef and vegetable row crop sources. This suggests that intervention steps for different pathogens may need to be addressed individually (IFSAC, 2015). Appropriately identifying the causative agent, food source, and contamination source will allow for more effective preventative measures to be developed and implemented to protect consumers.

1.6 Food and Drug Administration: Steps Toward a Safer Food Supply

1.6.1 Food and Drug Administration: Food Safety Modernization Act

On January 4, 2011, President Obama signed into law the FDA Food Safety Modernization Act (FSMA). FSMA marks a major turning point in food safety that was previously governed by the Federal Food, Drug, and Cosmetic Act (FD&C). FSMA allowed the FDA to enforce higher standards and regulatory procedures to achieve a safer food supply by focusing on implementing preventative measures (FDA, 2011). FSMA provided the FDA with rules and consequences that would aim to achieve high rates of

compliance with preventative measures, action plans, record keeping, and extensive safety measures to ensure standards are upheld by both domestic and foreign food suppliers. FSMA requires that all governed facilities write and maintain hazard analysis plans similar to Hazard Analysis Critical Control Point (HACCP) plans, and maintain extensive record keeping, to which the FDA may have access if necessary. FSMA holds the manufacturers and producers responsible for producing safe foods and food products. The law also gives FDA the power to hold foreign food producers to the same standards as domestic producers in an effort to protect the food supply at all levels (Gunawardhana & Czaban, 2013). The FDA is continuing research in an effort to enforce more rules such as the Produce Safety rule under FSMA to continue to improve all aspects of food supply.

1.6.2 The Produce Safety Rule

On November 13, 2015, the FDA finalized the Produce Safety Rule. The Produce Safety Rule was first proposed in January 2013, underwent strenuous revisions, and was re-proposed in September 2014 before being finalized in November 2015 and becoming effective January 26, 2016. The goal of this rule is to establish and apply science-based standards to all aspects of produce production to ensure its safety for human consumption (21 CFR § 11, 16, 112).

Under the Produce Safety Rule, two alliances have been formed to aid with education and guidance of farmers and producers. The first of these is the Produce Safety Alliance at Cornell University. The Produce Safety Alliance provides on-farm knowledge and assistance and develops tools for producer utilization to verify their compliance with the Produce Safety Rule. The second alliance is the Sprout Safety

Alliance at Illinois Institute of Technology. Because of recent outbreaks associated with fresh sprouts and the specific requirements outlined by the Produce Safety Rule, the Sprout Safety Alliance will be responsible for education, outreach, and aiding sprout producers with compliance to the Produce Safety Rule. These alliance programs will work in coordination with the FDA and USDA to ensure the Produce Safety Rule is understood, enforced, and maintained on all levels (FDA, 2015b).

1.6.2.1 Agricultural Water

The Produce Safety Rule contains several key aspects to protect produce from all angles. One notable aspect is the regulation on agricultural water. The final rule establishes quantitative criteria for water use during the production, harvest, and packaging of produce. The rule outlines two general criteria for generic *E. coli* counts in agricultural water.

> "The first rule states: No detectable generic *E. coli* are allowed for certain uses of agricultural water in which it is reasonably likely that potentially dangerous microbes, if present, would be transferred to produce through direct or indirect contact. Examples include water used for washing hands during and after harvest, water used on food contact surfaces, water used to directly contact produce (including to make ice) during or after harvest, and water used for sprout irrigation. The rule establishes that such water use must be immediately discontinued and corrective actions taken before re-use for any of these purposes if generic *E. coli* is detected. The rule prohibits use of untreated surface water for any of these purposes.
>
> The second set of numerical criteria is for agricultural water that is directly applied to growing produce (other than sprouts). The criteria are based on two values, the geometric mean (GM) and the statistical threshold (STV). The GM of samples is 126 or less CFU of generic *E. coli* per 100 mL of water and the STV of samples is 410 CFU or less of generic *E. coli* in 100 mL of water" (FDA, 2015b).

GM is a measure of the central tendency of your water quality distribution and is defined as the n^{th} root of the product of n numbers.

$$\bar{x}_{\text{geom}} = \sqrt[n]{\prod_{i=1}^{n} x_i} = \sqrt[n]{x_1 \cdot x_2 \cdot \ldots \cdot x_n}$$

STV is a measure of variability of your water quality distribution, derived as a model-based calculation approximating the 90th percentile using the lognormal distribution.

Due to the number of samples and complexity of calculations, UC Davis has developed an Excel spreadsheet tool that will allow the producers to input data in the format of CFU/100 mL and will calculate the GM and STV of their water samples. The spreadsheet, along with easy to follow instructions, can be found at http://ucfoodsafety.ucdavis.edu/files/229168.xlsx (UC Davis, 2016).

The Produce Safety Rule also outlines the rules and guidelines for the testing of agricultural water. The water testing requirements are based on the water source: surface or groundwater. The CDC (2009) defines surface water as water that collects on the ground or in a stream, lake, reservoir, or ocean. Groundwater is defined as water located below the ground surface in pores and spaces in the rock, typically accessed by drilling wells (CDC, 2009).

The rule considers untreated surface water to be at the highest risk of contamination. For this reason, untreated surface water that is to be applied to growing produce other than sprouts must have an initial survey using a minimum of twenty samples over the course of two or four years to calculate GM and STV. Once the initial survey is completed, five samples must be collected and tested annually. These five samples and the previous most recent fifteen samples will be used to calculate the GM

and STV to create a rolling database to ensure that the water quality is maintained (FDA, 2015b).

Untreated groundwater that is applied directly to growing produce, other than sprouts, is required to have an initial survey consisting of at least four samples collected over the period of one year to calculate GM and STV. Once the initial survey is complete, a minimum of one sample must be collected each year. Each annual sample plus the previous most recent three samples will be used to maintain a rolling database of GM and STV values to ensure the microbiological quality of the water is safe for agricultural use (FDA, 2015b).

Untreated groundwater that is to be used for any purpose for which no detectable generic *E. coli* is allowed, such as sprout irrigation and wash waters, is required to have an initial survey consisting of at least four samples over the period of a year. After the initial survey, testing is to be done at least once annually. The most recent four samples will be used to create a rolling database to verify the water is safe for use (FDA, 2015b).

1.6.2.2 Biological Soil Amendments

Another notable key aspect of the Produce Safety Rule is its regulations on biological soil amendments of animal origin. As defined by FSMA, soil amendments are any chemical, biological, or physical material intentionally added to the soil to improve the chemical or physical condition of the soil in relation to plant growth or to improve the capacity of the soil to hold water. Biological soil amendments of animal origin are biological soil amendments which consist, in whole or in part, of materials of animal

origin, such as manure or non-fecal animal byproducts, or table waste, alone or in combination.

Biological soil amendments of animal origin are of great concern because of their potential to contain pathogens of public health concern and their potential to contaminate crops if applied improperly (FDA, 2014). Guidelines and regulations for biological soil amendments of animal origin can be found in 21 C.F.R § 112.51. In these sections, the guidelines for classifying biological soil amendments of animal origin as treated or untreated are outlined. The use of human waste is prohibited. Requirements for the treatment, application, and harvesting of crops from biological soil amendments of animal origin are established with scientifically valid standards. Guidelines have also been established for proper record keeping in an effort to increase traceability in the event of a foodborne disease outbreak.

FSMA summarizes the biological soil amendment of animal origin regulations in table 1.

Table 1: 21 C.F.R. 112.56 states: You must apply the biological soil amendments of animal origin specified in the first column of the table in this paragraph in accordance with the application requirements specified in the second column of the table in this paragraph, and the minimum application intervals specified in the third column of the table in this paragraph.

If the biological soil amendment of animal origin is:	**Then the biological soil amendment of animal origin must be applied:**	**And then the minimum application interval is:**
Untreated…	In a manner that does not contact covered produce during application and minimizes the potential for contact with covered produce after application…	9 months

Untreated…	In a manner that does not contact covered produce during or after application…	0 days
(2) Treated by a scientifically valid controlled physical or chemical process, or combination of scientifically valid controlled physical and chemical processes, in accordance with the requirements of § 112.54(a) to meet the microbial standard in § 112.55(a)…	In any manner (i.e., no restrictions)…	0 days
(3) Treated by a scientifically controlled physical or chemical process, or combination of scientifically valid controlled physical and chemical process, in accordance with the requirements of § 112.54(b) to meet the microbial standard in § 112.55(b)…	In a manner that minimizes the potential for contact with covered produce during and after application…	0 days
(4)(a) Treated by a composting process in accordance with the requirements of § 112.54(c) to meet the microbial standard in § 112.55(b)…	In a manner that minimizes the potential for contact with covered produce during and after application…	45 days
(4)(b) Treated by a composting process in accordance with the requirements of § 112.54(c) to meet the microbial standard in § 112.55(b)…	In a manner that does not contact covered produce during or after application…	0 days

1.7 Summary

Microbial contamination of the environment has the potential to increase the risk of contamination of fresh produce being grown within that environment. Understanding the impact of animal agriculture on microbial environmental quality and the impact of

environmental quality on food safety will help to identify areas of production that can be improved upon.

Evaluating all aspects of animal and crop production can provide information for the development of better management practices. FSMA has made a large impact on food safety by developing and implementing the Produce Safety Rule. FSMA and the Produce Safety Rule focus heavily on preventative measures rather than reactive measures. By implementing science-based methods of microbial monitoring, surveillance, and recordkeeping, it may be possible to minimize the likelihood of contaminated crops reaching tables around the United States.

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Chapter 2: The transfer and persistence of *Salmonella* spp. in an animal production environment

2.1 Introduction:

Salmonella spp., the leading bacterial cause of foodborne illness in the United States, saw a 39% increase from 2012 to 2013 (CDC, 2015). *Salmonella* spp. outbreaks have typically been associated with the consumption of undercooked poultry, eggs, and egg products. However, in recent years, *Salmonella* spp. outbreaks have been increasingly associated with other food sources such as beef, pork, and fresh produce (FDA Bad Bug Book, 2012). By combining animal surveillance data and food attribution data, a correlation between many *Salmonella* serotypes and specific reservoirs have been seen, but this is not an absolute. Some *Salmonella* serotypes are associated with multiple hosts, and those with environmental, amphibian, or reptile reservoirs might be more likely to be transmitted by fresh produce (Jackson, Griffin, Cole, Walsh, & Chai, 2013).

Salmonella Typhimurium has been shown to infect various hosts and has the ability to survive in the environment for long periods of time. Contaminated pastures have been shown to serve as a secondary host and reservoir for the transmission and infection of new animals when exposed to the environment (Jensen, Dalsgaard, Stockmarr, Nielsen, & Baggesen, 2006).

Understanding the environment's role in the spread of foodborne pathogens, this study was designed to evaluate the presence and persistence of *Salmonella* spp. in the environment. By detecting experimental Nalidixic acid resistant strains of *Salmonella* Heidelberg and *Salmonella* Enteritidis and monitoring its spread in the environment

surrounding the Auburn University Poultry Research Unit, it can be determined that farm sanitation practices do need to be improved upon.

2.2 Materials and Methods:

2.2.1 Collection Procedures:

The Auburn University Poultry Research Farm Unit and surrounding area was selected for environmental sampling for detection of *Salmonella spp.* The first round of weekly samples was collected on July 14, 2015; July 23, 2015; July 32, 2015; August 10, 2015; and August 20, 2015 and consisted of six sampling sites on and around the farm. Each site was selected based on distance from the farm entrance (Figure 2) and type of sample (solid vs. water) (Table 2). The litter barn (A), two runoff lagoons (B, C), and two ponds (D, E) were sampled on the Auburn University Poultry Research Farm Unit. One sampling site (F), not located on the AU Poultry Farm but in close proximity, was selected to determine if *Salmonella spp.* could be detected in the areas surrounding the farm.

A second round of weekly sampling took place September 24, 2015; October 1, 2015; October 8, 2015; and October 23, 2015. Each sampling site from the first round (A through F) was sampled along with seven additional sites. Each of the additional sites were located outside of the farm's perimeter (G-M) (Figure 2).

Table 2: Map identification corresponding to Figure 2, type of sample collected, and the distance from the unit entrance for each sampling site

Map ID	Type of sample collected	Distance from entrance (km)
Star		0 km
A	Used Litter	0.13
B	Water	0.15
C	Water	0.18
D	Water	0.26
E	Water	0.27
F	Soil	0.35
G	Soil	0.13
H	Soil	0.38
I	Soil	0.41
J	Soil	0.09
K	Soil	0.44
L	Water	0.51
M	Soil	0.52

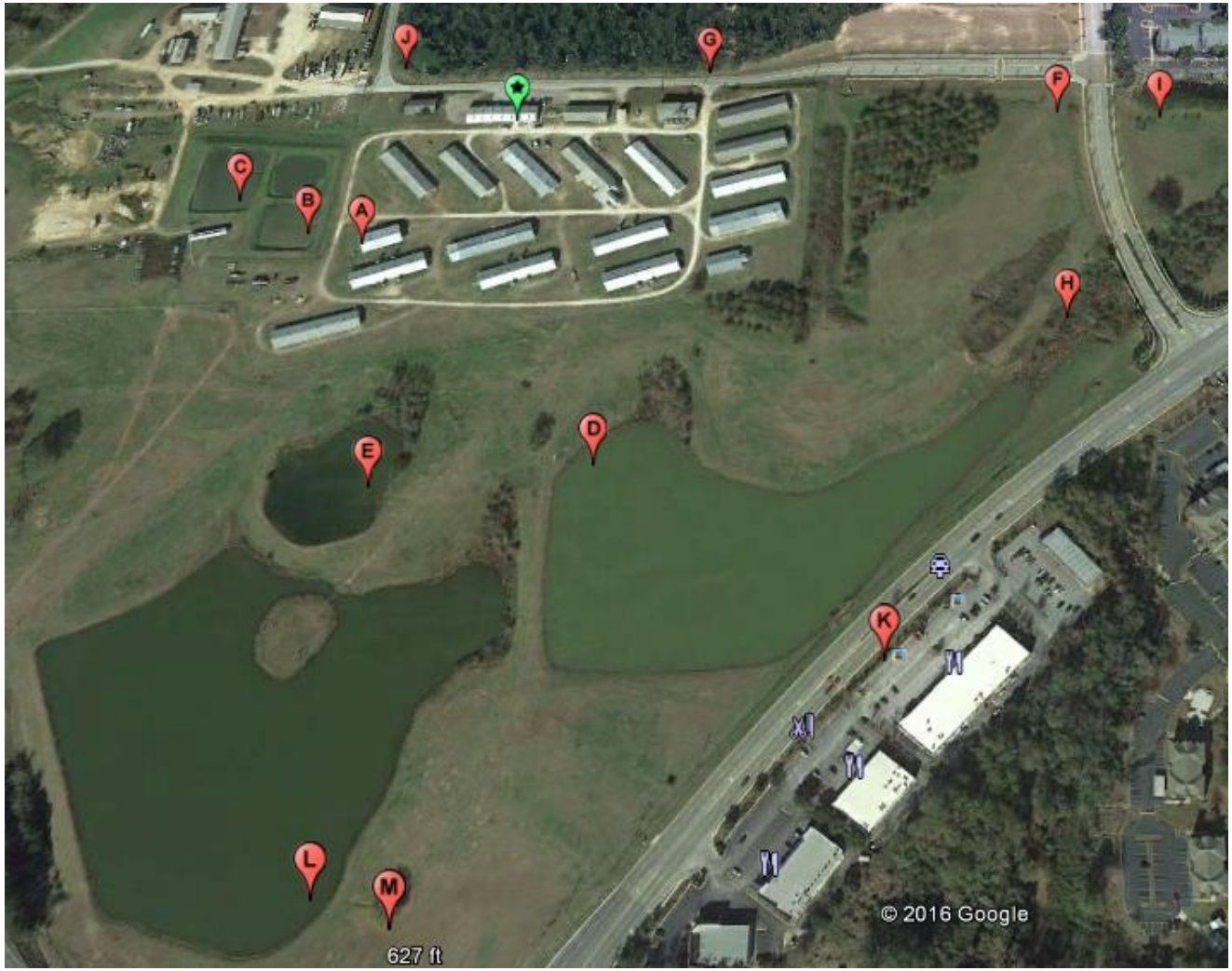

Figure 2: Map of Auburn Univeristy Poultry Research Unit. Green star indicates the entrance to the unit. This is the point from which all sampling sites A-M were measured.

2.2.2 Sampling Methods

For aquatic locations, 500 mL of water was collected in a sterile 1 L glass bottle. At the litter barn, litter samples from each quadrant of the barn were collected to total 250 g and placed in a sterile Whirl-Pak® bag (Nasco, Fort Atkinson, WI, USA). Locations at which soil samples were collected, 250 g of soil and plant debris was collected and placed in a sterile Whirl-Pak® bag. All of the samples were placed into a cooler with ice and transported to the lab for further processing.

2.2.3 Pre-enrichment using Buffered Peptone Water (BPW):

Upon arrival at the lab, each of the water samples were pre-enriched by adding 500 mL BPW (BD/Difco Laboratories, Sparks, MD, USA) to each of the jars. For the litter and soil samples, 500 mL BPW was added to each Whirl-Pak® bag. The samples were mixed thoroughly by hand shaking and placed in the incubator at 37°C ±0.5°C for 24 hours ±2 hours.

2.2.4 *Salmonella spp.* Enrichment using Tetrathionate broth and Rappaport Vassiliadis broth:

After pre-enrichment, the samples were removed from the incubator and hand shaken for two minutes to evenly distribute the bacteria. Each of the samples were then enriched for *Salmonella spp.* utilizing Tetrathionate (TT) broth (BD/Difco Laboratories, Sparks, MD, USA) and Rappaport Vassiliadis (RV) broth (BD/Difco Laboratories, Sparks, MD, USA). Five hundred microliters of each sample was transferred to a tube containing 10 mL of TT broth, and 100 µL of each sample was transferred to a tube

containing 10 mL of RV broth. The TT and RV broth tubes were loosely capped and incubated at 41°C ±2°C for 24 hours ± 2 hours.

2.2.5 Isolation of suspect *Salmonella spp.* colonies via XLT 4 agar:

After incubation at 41°C ±2°C for 24 hours ± 2 hours, the tubes were removed from the incubator and mixed via vortex mixer. Using a sterile 10 µL disposable loop, samples from each broth tube were streaked onto XLT4 agar (BD/Difco Laboratories, Sparks, MD, USA) plates for colony isolation. The plates were incubated at 37°C ±0.5° for 24 to 48 hours. If no suspect *Salmonella* colonies were observed at 24 hours, they were allowed to incubate for an additional 24 hours then observed again. Black top, suspect *Salmonella* colonies were picked from the XLT4 plates and re-streaked onto XLT4 for isolation. Pink-yellow colonies were also selected as suspect Hydrogen Sulfide (H_2S) negative *Salmonella* colonies and re-streaked onto XLT4 plates for isolation. These plates were then incubated at 37°C ±0.5°C for 24 to 48 hours. An isolated colony was then selected from each plate and transferred to a Trypticase Soy Agar (TSA) (BBL/Difco Laboratories, Sparks, MD, USA) plate. The TSA plates were incubated at 37°C ±0.5°C for 24 to 48 hours. After incubation, the samples were transferred to Auburn University College of Veterinary Medicine for further screening and confirmation.

2.2.6 Confirmation of *Salmonella spp*. from culture:

Lysine Iron Agar (LIA) slants (BD/Difco Laboratories, Sparks, MD, USA), Triple Sugar Iron Agar (TSI) slants (BD/Difco Laboratories, Sparks, MD, USA), and Urea agar slants (BD/Difco Laboratories, Sparks, MD, USA) were made according to

manufacturer's instructions. One colony from each TSA plate was picked to inoculate slants of LIA, TSI, and Urea by stabbing to the butt and streaking over the slant. The LIA, TSI, and Urea slants were incubated, with loosened screw caps, at 35°C ± 2°C for 24 ± 2 hours. After incubation, each of the slants were evaluated for media changes. *Salmonella* positive LIA slants should have a purple slant with a black butt signifying H_2S production. H_2S negative *Salmonella spp.* will have an LIA slant that is purple throughout. Hydrogen Sulfide producing *Salmonella* positive TSI slants will produce a red slant with a black butt. Hydrogen Sulfide negative *Salmonella* will produce TSI slants that have a red slant and a yellow butt. *Salmonella* is urease negative, so the urea test will have no change, and agar will remain yellow in color. The samples with the expected results for all three LIA, TSI, and Urea tests were confirmed as *Salmonella* and were later serogrouped.

2.2.7 Creating freezer stock for later analysis:

The colonies used to for LIA, TSI, and Urea slants were also used to create a freezer stock for further analysis at later times. The colony was used to inoculate 3 mL of Luria-Bertani (LB) broth and incubated at 35°C ± 2°C for 24 ± 2 hours. A sterile swab was used to transfer cells from the LB broth to an LB agar slant. The LB agar slants were incubated at 35 ± 2°C for 18-24 hours. After incubation, 0.5 mL 1X PD buffer was added to the slant with overnight growth. The slants were then vortexed for 30 to 60 seconds to wash the slant growth down into the 1X PD buffer. Then, 0.2 mL of 1X PD buffer with cells was removed and added to 0.8 mL of 100% glycerol in a 1.8 mL cryotube. The cryotubes were vortexed vigorously and inverted multiple times to ensure proper mixing of the cells and glycerol. The tubes were then stored at -20°C. Working

TSA slants of freezer stock were made by using a sterile disposable 1 µL loop to transfer the *Salmonella spp.* cells to a new TSA slant. The slants were incubated at 35 ± 2°C for 24 hours with loosened screw caps.

2.2.8 Serogrouping Analysis:

The samples that were confirmed to be *Salmonella* were then serogrouped using Difco™ *Salmonella* Antiserum. To prepare each sample to be serogrouped, the freezer stock was used to create workable TSA slants. After workable TSA slants were incubated, a sterile disposable 10 µL loop was used to transfer a loop of cells into a tube containing 3 mL of Buffered Peptone Water and incubated at 35 ± 0.5°C for 24 ± 2 hours. Using a 1 mL pipette, one drop of broth sample containing *Salmonella spp.* cells was applied to a glass agglutination plate. One drop of antisera was added to each drop of the sample, mixed, and observed for agglutination. Each isolate's poly group was first determined by testing for agglutination using Poly A (groups A, B, D, E_1, E_2, E_3, E_4, L), Poly B (groups C_1, C_2, F, G, H), Poly C (groups I, J, K, M, N, O), and Poly D (groups P, Q, R, S, T, U). Once the poly group was determined, the serogroup was determined by using antiserum for Group B, Group C1 (factors 6, 7), Group C2 (factors 6, 8), Group D1 (factors 1, 9, 12), Group D2 (factors 9, 46), Group E (factors 1, 3, 10, 15, 19, 34), Group K (factor 18), or Group O (factors 4, 5) (BD/Difco Laboratories, Sparks, MD, USA).

2.2.9 Serotyping and Pulse Field Gel Electrophoresis (PFGE) at National Veterinary Service Laboratory in Ames, Iowa:

Because of the prevalence, samples that had positive agglutination tests for serogroup B were selected to be sent for serotyping. To prepare samples for serotyping, a

sterile disposable 1 µL loop was used to transfer cells from the freezer stock to a 5 mL TSA slant. The slants were incubated at 35 ± 0.5°C for 24 ± 2 hours with loosened screw caps. After incubation, the samples were sent to the National Veterinary Service Laboratory in Ames, Iowa for serotyping.

After serotyping, samples identified as *Salmonella* Saintpaul were submitted for PFGE to determine genetic similarities.

2.3 Results

Environmental sampling at the Auburn University Poultry Research Farm Unit detected several different serovars of *Salmonella* spp. in each sampling location. A separate inoculation and necropsy study at the Poultry Research Unit was using Nalidixic acid resistant *Salmonella* Enteritidis and *Salmonella* Heidelberg during this time period. Because of the known origination and the Nal resistant characteristic, detection of this isolate could easily be traced. An inoculation with Nalidixic acid resistant *S.* Enteritidis occurred on July 9, 2015. The necropsy of those chicks occurred on September 10, 2015.

The first round of weekly sample collection and analysis began July 14, 2015 and ended on August 20, 2015 (Table 3). Sampling sites A through F were collected on the dates indicated (Figure 3). On July 14, Nalidixic acid resistant *S.* Enteritidis was isolated from B and C. On July 23, *Salmonella* spp. belonging to group D was isolated from location A, and *Salmonella* spp. belonging to poly group D were isolated from locations E and F. On July 31, group D was isolated from location A and group B from locations B, C, and E. After the necropsy of experimental chicks on August 10, Nalidixic acid resistant *Salmonella* was isolated from location B that same day. *Salmonella* spp.

belonging to group C2 was isolated from location C and poly group D from location F. On August 20, group B *Salmonella* spp. were isolated from locations B, C, E, and F. Group C2 *Salmonella* spp. were isolated from location D and poly group C from location A.

Because *Salmonella* spp. were detected at all locations on and around the perimeter of the farm, a second round of weekly sampling took place September 24, 2015 until October 24, 2015. Studies involving *Salmonella* Heidelberg and *Salmonella* Enteritidis were being conducted at the Poultry Research Unit during this time. Chicks were inoculated with Nalidixic acid resistant *S.* Heidelberg on August 28 and Nalidixic acid resistant *S.* Enteritidis on September 22. Necropsy of chicks with Nal resistant *S.* Heidelberg occurred on September 28.

The original locations (A-F) (Table 4) were sampled along with 7 additional locations (G-H) (Table 5). Nalidixic acid resistant *S.* Enteriditis (D1) was detected at locations A, B, C, H, and K. Nalidixic acid resistant *S.* Heidelberg (B) was isolated from locations A and C on October 23. *Salmonella* spp. from groups C1, C2, D1, E and poly groups A, B, D, and E were isolated throughout the study (Figure 3).

Nalidixic acid sensitive *Salmonella* spp. belonging to serogroup B was isolated from 7 out of 13 sampling locations and on 4 out of 9 sampling dates. Nalidixic acid resistant *Salmonella* spp. were isolated from locations A and C on October 23. Each of the samples belonging to serogroup B were sent to the USDA National Veterinary Services Laboratory in Ames, Iowa for serotyping. The two Nalidixic acid resistant samples were confirmed to be the experimental strain of *Salmonella* Heidelberg being

used at the Poultry Research Unit. All other serogroup B samples were identified as *Salmonella* Saintpaul (Figure 4).

Pulse Field Gel Electrophoresis (PFGE) was performed on all *S.* Saintpaul samples. All samples produced the same banding pattern (Figure 5). These results demonstrate that each of these isolates is genetically identical and belonging to the same strain, further strengthening the idea that vectors and/or environmental factors may be contributing to the spread of *Salmonella* spp. in the environment.

Table 3: Serogroup results from first round of weekly sampling. Locations A through F were sampled on the dates indicated in the left column. Each serotype detected is color coded to match the distribution map in Figure 2.

	A	B	C	D	E	F
9-Jul	Chicks innoculated with Nalidixic Acid resistant *Salmonella* Enteritidis					
14-Jul		D1	D1			
23-Jul	D				Poly D	Poly D
31-Jul	D	B	B		B	
10-Aug	Necropsy of chicks with Nalidixic Acid resistant *Salmonella* Enteritidis					
10-Aug		D1	C2			Poly D
20-Aug	Poly C	B	B	C2	B	B

Table 4: Serogroup results from second round of weekly sampling for locations A through F. Sampling dates are indicated in the left column. Each serotype detected is color coded to match the distribution map in Figure 2.

	A		B		C				D			E		F			
28-Aug	Chicks innoculated with Nalidixic Acid resistant *Salmonella* Heidelberg																
22-Sep	Chicks innoculated with Nalidixic Acid resistant *Salmonella* Enteriditis																
24-Sep			Poly A	C1	C1	Poly A			B	C2	C1	B	C1	Poly D	Poly A	Poly B	C1
28-Sep	Chicks with Salmonella Heidelberg necropsied																
1-Oct			D1	E	D1	E			Poly C			B	Poly B	E			
8-Oct	D1		C2		D1	E	D1	Poly A	Poly C			unknown					
23-Oct	C1	B	D1		B				Poly A					Poly A			

Table 5: Serogroup results from second round of weekly sampling for locations G through M. Sampling dates are indicated in the left column. Each serotype detected is color coded to match the distribution map in Figure 2.

	G	H		I		J			K					L		M
28-Aug	Chicks innoculated with Nalidixic Acid resistant *Salmonella* Heidelberg															
22-Sep	Chicks innoculated with Nalidixic Acid resistant *Salmonell* a Enteriditis															
24-Sep		Poly A	C2	C2		B	C1	C2	C2	Poly A				Poly A	C2	B
28-Sep	Necropsy of chicks with Nalidixic Acid resistant Salmonella Heidelberg necropsied															
1-Oct	Poly D	D1	C2	C2	Poly B	B			C2	B	D1	Poly E	D1			B
8-Oct						unknown	Poly A		Poly D							D1
23-Oct		D1		D1												D1

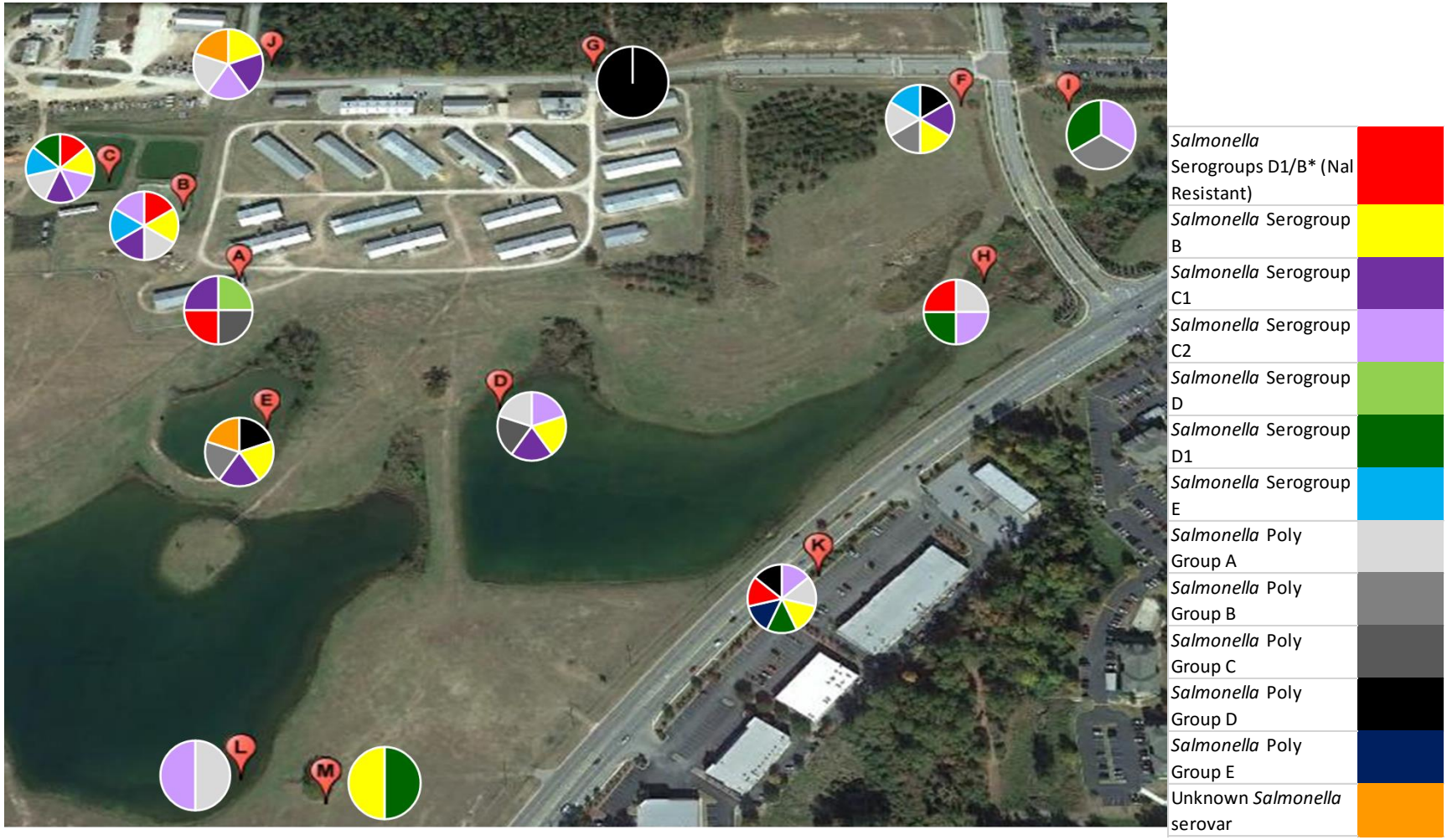

Figure 3: Map of Auburn University Poultry Research Unit. Sampling locations are labeled A-M. Proportions of each color do not correspond to concentrations of each Salmonella spp. at that location.

Figure 4: Map of Auburn University Poultry Research Unit. Yellow stars indicate sites at which *Salmonella* Saintpaul was confirmed using serotyping.

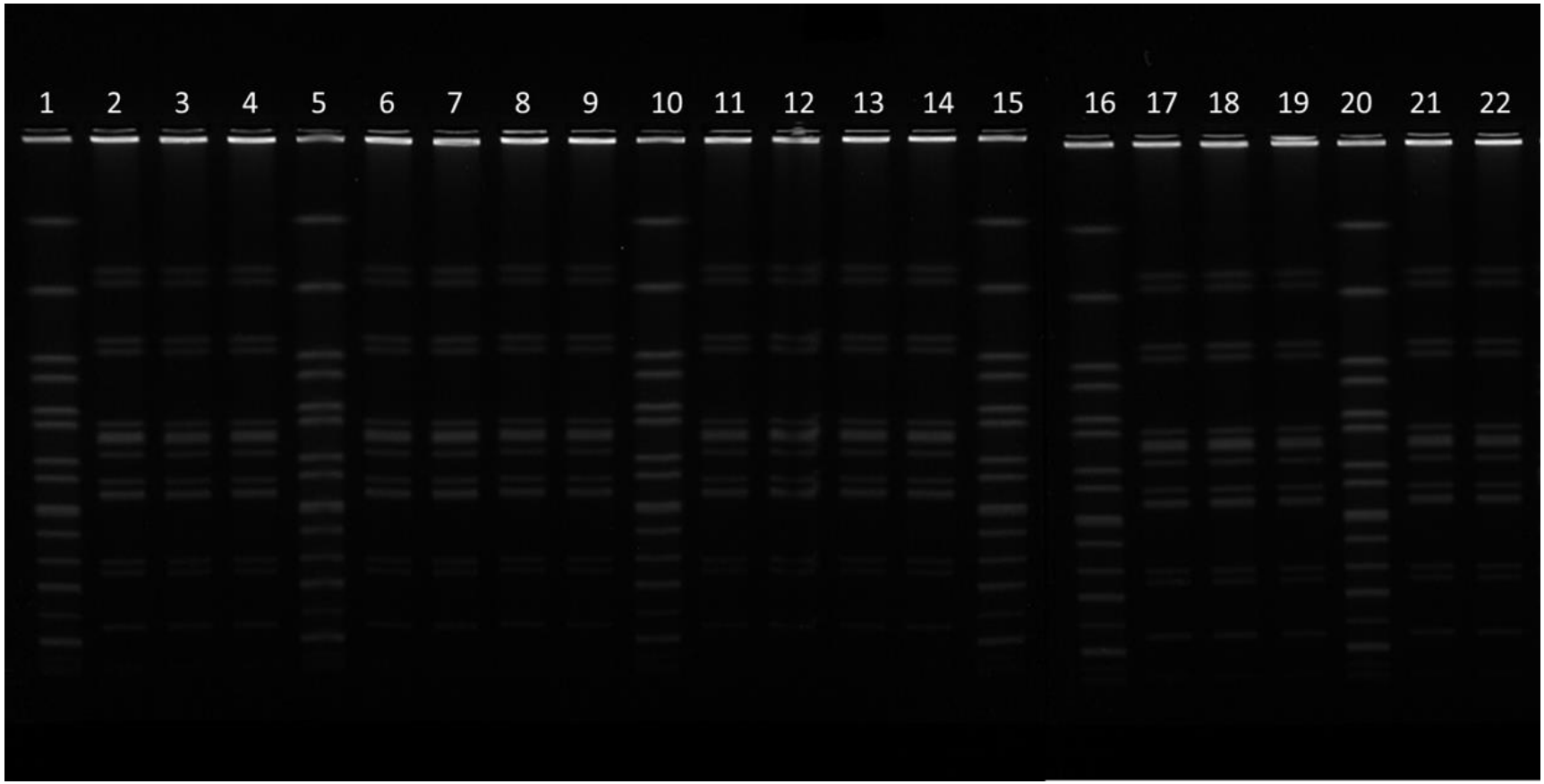

Figure 5: PFGE analysis results for *Salmonella* Saintpaul isolates. Lanes 1, 5, 10, 15, 16, and 20 are ladders. All other lanes contain *S.* Saintpaul samples.

2.4 Discussion

2.4.1 Detection of Nalidixic acid resistant *Salmonella* Enteritidis and Heidelberg

For the initial detection of Nalidixic acid resistant *Salmonella* Enteritidis and Heidelberg, results showed that these strains can be detected in the environment around the poultry houses. Chicks were inoculated with the experimental Nalidixic acid resistant strains on July 9, 2015, necropsied on August 10, 2015, and were housed within the poultry houses and not directly exposed to the environment. The initial round of weekly environmental sampling occurring on July 14, July 23, July 31, August 10, and August 20 revealed the detection of Nalidixic acid resistant *Salmonella* spp. in locations B and C. These two locations are described as runoff lagoons which collect wash water and debris from the inside of the poultry houses. Location B is the first lagoon to which this runoff water is deposited. Location C is an overflow lagoon that receives water from location B when water levels rise.

A second round of weekly sampling took place on September 24, October 1, October 8, and October 23. During this time, chicks were inoculated with Nalidixic acid resistant *Salmonella* Heidelberg and Enteritidis on September 28 and October 22, respectively. After the September 28 necropsy of chicks that had been inoculated with Naladixic acid resistant *Salmonella* Heidelberg, an increase in Nalidixic acid resistant *Salmonella* spp. was detected in the environmental samples. Because of the results from the initial survey, it was expected that Nalidixic acid resistant *Salmonella* spp. would likely be detected at locations B and C. The additional detection of Nalidixic acid resistant *Salmonella* spp. at location A was not surprising, because this is the location at

which used litter and fecal material are deposited for holding. Nalidixic acid resistant *Salmonella* spp. were also detected in locations H and K. These two locations are not considered to be on the farm, and do not have direct contact with equipment or individuals from the farm. There is a visually observed downward slope of the landscape from the poultry farm toward these locations. Because runoff water has been shown to play a major role in the movement of microbes from agricultural environments (Hubbard, Newton, & Hill, 2004), it is likely that rainwater runoff served as a means of spreading *Salmonella* spp. from the farm to these locations. The physical barrier of a four lane road between the farm and location K did not prevent the movement of *Salmonella* spp. off the farm. Numerous storm drains run under this road, and contamination of runoff water may potentially lead to the contamination of several water systems.

2.4.2 Distribution and Persistence of *Salmonella* spp. serogroups in the environment

Distribution of *Salmonella* spp. on and around the Auburn University Poultry Research Unit was evaluated to determine if *Salmonella* spp. were potentially spreading in the environment. At the conclusion of sample collection, it was noticed that *Salmonella* serogroups B and C2 were isolated most frequently and from the most sampling sites. Serogroup B was isolated from eight sampling sites on four different days. Serogroup C2 was isolated from eight sampling sites on 5 separate days. Both serogroups displayed similar distributions, and samples that were identified as serogroup B were chos to be submitted for serotyping. All samples were positively identified as *Salmonella* Saintpaul at locations B, C, D, E, F, J, K, and M. This suggests that vectors or environmental factors may be contributing to the spread of *Salmonella* throughout the environment. To determine if these *S.* Saintpaul isolates are genetically identical, Pulse

Field Gel Electrophoresis (PFGE) was performed. Confirming that these isolates were genetically identical, demonstrates that *Salmonella* spp. are being spread throughout the environment. Knowing *Salmonella* spp. have the potential to spread throughout the environment presents the issues of cross-species, run-off water, surface water, and crop contamination.

It has already been discussed that rainfall and runoff water can contribute to the movement of *Salmonella* spp. around the environment. Other contributing factors may include farm equipment, workers, and wildlife. On every sampling date, numerous turtles could be observed in each location at which aqueous samples were collected. Other animals such as wild birds, rodents, and insects may also contribute to the movement of microbes around the environment. These are all plausible vectors of transmission in this situation (Winfield & Groisman, 2003).

It is not uncommon to find *Salmonella* spp. in the environment, but the long term survivability varies among serotypes (Jackson et al., 2013). Over the course of four months, sampling sites A through F were sampled a total of nine times during two separate one month periods. Sampling sites G through M were sampled weekly over the course of one month. Within this time period, several different *Salmonella* serogroups were detected at each location. The most evident persistence was noticed at sampling site E in which *Salmonella* serogroup B was detected on four out of nine collection dates. While Nalidixic acid resistant *Salmonella* serogroup D1 was isolated from location B four out of nine dates, this is likely due to recontamination after inoculation and necropsy of experimental chicks.

2.5 References

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Chapter 3: Evaluation of surface water quality on animal agriculture farms

3.1. Introduction

Over the past few decades, the demands for crop and livestock production have seen a large increase. The result is an increase in environmental contaminants entering surface water sources. Agricultural NPS pollution has been identified as the leading cause of river and stream water quality deterioration. Runoff water from agricultural land plays a major role in the contamination of water sources. As rainfall or snowmelt flows over and through the ground, animal feces and soil particles with associated microbes are washed away and deposited into streambeds, lakes, rivers, ponds, and other water reservoirs (CDC, 2010).

Three-fourths of Alabama farms use surface water sources including ponds, lakes, and streams for irrigation purposes. Some farmers have the ability to harvest winter and spring water to store for use during summer irrigation (Curtis & Rochester, 1994). Utilizing surface water and water harvesting can be an efficient method for ensuring sufficient water supply is available, but there are known microbial risks of using these water sources for crop irrigation (FDA, 2015).

Rainfall is known to be one of the main stressors of surface water quality. Evaluation of microbial water quality of the Göta Älv river in Sweden showed a strong positive correlation between heavy rainfall and *E. coli* concentrations observed in the surface water of the river (Tornevi, Bergstedt, & Forsberg, 2014). The microbial quality of two fresh-water creeks in North Carolina was evaluated before and after rainfall. It was determined that runoff water from urban, suburban, and rural land resulted in an

increase of fecal coliform concentrations along with other pollutants (Mallin, Johnson, & Ensign, 2009). Irrigation water has been shown to serve as a source of microbial contaminants for crop production. Solomon et al. demonstrated that the use of irrigation water contaminated with *E. coli* O157:H7 resulted in the contamination of the edible portion of lettuce plants.

An increase in produce related foodborne illness outbreaks has gained the attention of food regulatory agencies such as the FSMA. With the introduction of FSMA in 2011, food safety focus has been shifted toward implementing more preventative measures throughout the food supply chain. In 2015, FSMA released the Produce Safety Rule to be implemented in January 2016. Under the Produce Safety Rule, farmers are now required to comply with new regulations. Agricultural water and biological soil amendments are now subject to FDA regulations in an effort to prevent crop contamination. Generic *E. coli* must be monitored in irrigation water, biological soil amendments must be properly treated and applied, and proper recordkeeping is being enforced for crop producers.

The impact that this rule may have on producers has not been evaluated, and the uncertainty of the effects raise many concerns with producers when it comes to irrigation water quality. Based on these concerns, this study was designed to evaluate the impact of animal agriculture on the surface water quality of water sources from areas with varying degrees of animal agriculture. Data was collected and analyzed according to the requirements outlined by the Produce Safety Rule and the use of the provided Excel tool by the Cornell University Produce Safety Alliance.

3.2 Materials and Methods

3.2.1 Field Sampling Locations and Methods

Six farms in East Central Alabama were selected as candidates for stream water and/or pond water sampling. Three of these farms contained both streams and ponds. One farm contained only a stream, and the other two contained only a pond. Water from the streams was collected where the stream entered the farm and where the stream exited the farm. From the ponds, surface water and sediment samples were collected. The farm identification and type of water samples collected at each is as follows:

A: Stream in, stream out.
B: Stream in, stream out, pond
C: Stream in, stream out, pond
D: Stream in, stream out, pond
E: Pond
F: Pond

All samples were collected between 9:00 am and 1:00 pm on the days sampled. The water from each of the streams was collected at the site where they enter the farm and the site where they exit the farm. Each surface water stream sample was collected by dipping a sterile 500 mL glass bottle into the water approximately 6-12 inches below the water surface. The bottles were tightly capped, labeled, and stored on ice during transport from the farms to Auburn University for laboratory analysis. Stream sampling took place once every season; Fall on November 18, 2015; Winter on February 22, 2016; Spring on April 13, 2016; and Summer on June 20, 2016.

Pond water was collected by wading out and using a telescopic collection pole and cup (Ben Meadows ® Company, Janesville, WI, USA) to collect undisturbed surface

water 6-12 inches below the water surface. The water was immediately poured into a sterile 500 mL glass bottle, labeled, and stored on ice for transport. Pond surface water samples were collected monthly for 9 consecutive months.

3.2.2 Surface Water Analysis using EPA Method 1603:

Analysis utilizing modified-mTEC agar (BD/Difco Laboratories, Sparks, MD, USA) was conducted according to EPA Method 1603 to analyze in stream surface water, out stream surface water, and pond surface water. Modified mTEC agar was prepared according to the manufacturer's instructions. Each of the petri dishes were labeled with the sample identification and volume of water to be filtered. Each sample was vigorously shaken to distribute the bacteria uniformly. PALL MicroFunnel™ ST Filter Units (Life Sciences, Ann Arbor, MI, USA) with a 0.45 µm pore size were attached to the filter base, and 3 mL, 10 mL, and 30 mL aliquots of each sample were measured out and added to separate filter funnels. The samples were filtered using a vacuum, and the sides of each of the funnels was rinsed using 20 mL sterile water. After rinsing, the vacuum was turned off and the funnel was removed from the filter base. Using sterile forceps, each membrane filter was removed from the base and carefully rolled onto the modified mTEC agar being careful to avoid trapping any air bubbles between the filter and the agar. The petri dish was closed, inverted and incubated at 35°C ± 0.5°C for 2 ± 0.5 hours to resuscitate any stressed bacteria. After incubation at 35°C ± 0.5°C for 2 ± 0.5 hours, the plates were transferred to Whirl-Pak® bags. A maximum of 25 plates were placed in each Whirl-Pak® bag. The Whirl-Pak® bags were sealed and submerged in a 44.5°C ± 0.2°C waterbath for 22 ± 2 hours. After 22 ± 2 hours, the bags and plates were removed

from the waterbath. Red or magenta colored colonies were counted and recorded as *E. coli* colonies.

3.3 Results

Water samples were collected and evaluated according to US EPA method 1603. Countable plates were considered plates with 20-80 *E. coli* colonies. For samples with more than one countable plate, the average of all countable plates was used. For samples in which all plates were above or below 20-80 *E. coli* colonies, the plate closest to the range was used. Plates were calculated to CFU/100 mL, then that value was input to the UC Davis Western Center for Food Safety Excel Tool for untreated surface water provided at http://wcfs.ucdavis.edu/. This Excel tool, developed by the Produce Safety Alliance, helps producers monitor the microbiological water quality profile (MWQP) for their surface water source. Once the data has been input for each sample, the Excel tool calculates the GM and STV for each sample and notifies the producer as to whether or not the water meets the Produce Safety Rule criteria. If the values do not meet the criteria, the tool will provide how many days are necessary between last irrigation and harvest if using microbial die-off method.

3.3.1 Farm A:

Farm A is described as a 250-acre cow-calf operation with approximately 180 head of cattle. The sampled stream runs directly through the pastures, and the cattle have free access to the water. Four quarterly samples were collected at both the entrance point and the exit point (Figure 6). USEPA method 1603 was used to enumerate generic *E. coli*/100 mL for each sample (Figure 7). Using these values, Geometric Mean (GM) and

Statistical Threshold Value (STV) were calculated for both the entrance (table 6) and exit (Table 7) points of the stream.

The entrance point has a GM value of 357 generic *E. coli*/100 mL and an STV value of 592 generic *E. coli*/100 mL. The exit point has a GM value calculated at 256 generic *E. coli*/100 mL and an STV value calculated at 2060 generic *E. coli*/100 mL. Based on the values calculated from these four values, neither point of this stream meets the Produce Safety Rule criteria for irrigation water. Both the entrance and exit points of this stream would require at least a one-day period between last irrigation and harvest for microbial die-off.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 6: Layout of farm A: Stream entrance sampling site denoted by a green star and stream exit sampling site denoted by red octagon.

Table 6: Farm A stream entrance GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	357	2.55	592	2.77
Deviation from criteria		0.45		0.16
Does your water meet PSR criteria?		No		No
Are corrective measures necessary?		Yes		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		1		1

Table 7: Farm A stream exit GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	256	2.41	2060	3.31
Deviation from criteria		0.31		0.70
Does your water meet PSR criteria?		No		No
Are corrective measures necessary?		Yes		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		1		2

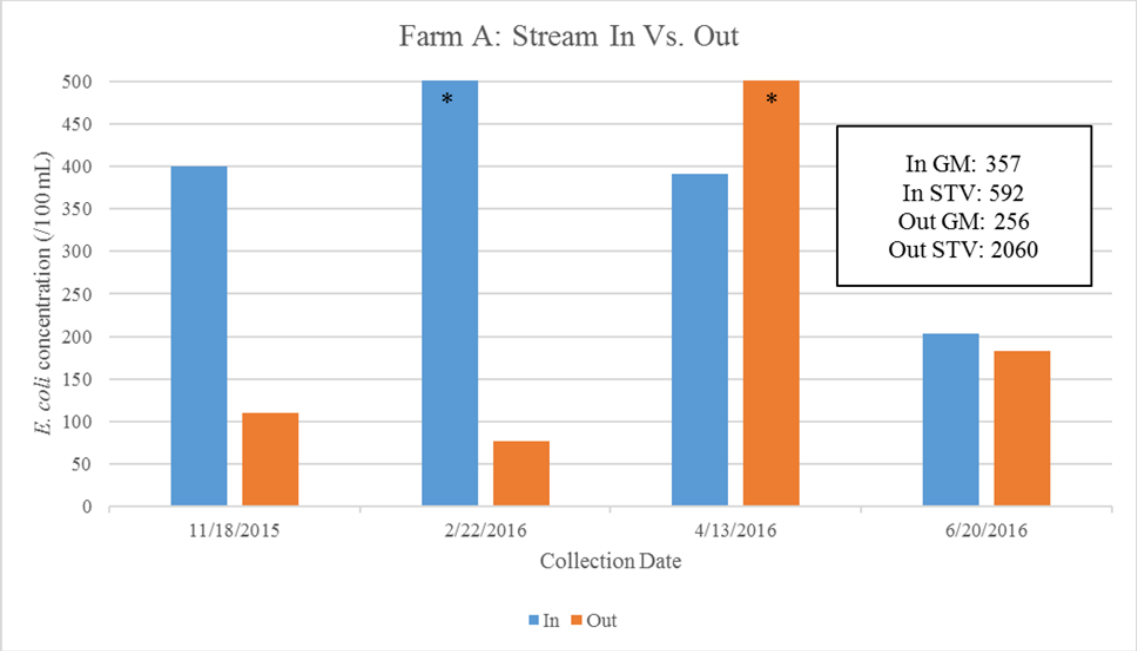

Figure 7: Generic E. coli concentrations in water samples collected from stream entrance and exit points on farm A.
*denotes E. coli concentrations greater than 500 CFU/100 mL

3.3.2 Farm B:

Farm B is described as a 400-acre cow-calf operation with approximately 150 head of cattle. The cattle have free access to the stream, which runs along the inside perimeter of the farm, and the pond (Figure 8). Four quarterly surface water samples were collected at the entrance and exit points of the stream; nine monthly surface water samples were collected from the pond. USEPA method 1603 was used to enumerate generic *E. coli*/100 mL for each entrance and exit samples (Figure 9) and each pond sample (Figure 10). Using these values, GM and STV were calculated for the stream entrance (Table 8), stream exit (Table 9), and pond (Table 10).

The stream entrance GM and STV results were 614 and 993 generic *E. coli*/100 mL, respectively. Stream exit GM and STV results were 334 and 574 generic *E. coli*/100

mL, respectively. GM and STV results for the pond samples were 16 and 78 generic *E. coli*/100 mL, respectively.

Based on these criteria, neither the entrance nor the exit points of the stream satisfy the requirements for the Produce Safety Rule for irrigation microbial water quality. The entrance point of this stream would require a two-day die-off period, and the exit point would require a one-day die-off period. The pond, on the other hand, meets the requirements for generic *E. coli* counts for irrigation water, and no corrective actions are necessary at this point in time.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 8: Farm B stream entrance (green star), stream exit (red octagon), and pond (yellow triangle) sampling sites.

Table 8: Farm B stream entrance GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	614	2.79	933	2.97
Deviation from criteria		0.69		0.36
Does your water meet PSR criteria?		No		No
Are corrective measures necessary?		Yes		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		2		1

Table 9: Farm B stream exit GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	334	2.52	574	2.76
Deviation from criteria		0.42		0.15
Does your water meet PSR criteria?		No		No
Are corrective measures necessary?		Yes		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		1		1

Table 10: Farm B pond GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	16	1.21	78	1.89
Deviation from criteria		-0.89		-0.72
Does your water meet PSR criteria?		Yes		Yes
Are corrective measures necessary?		No		No
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		0

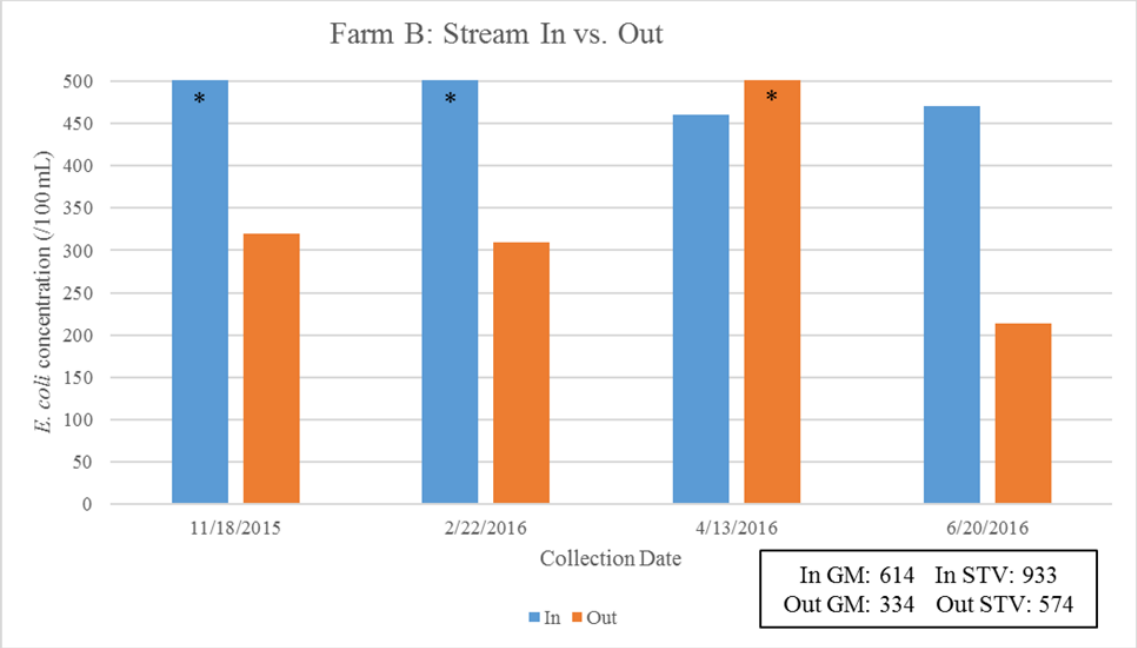

Figure 9: Generic *E. coli* concentrations in water samples collected from stream entrance and exit points on farm B.
*denotes *E. coli* concentrations greater than 500 CFU/100 mL

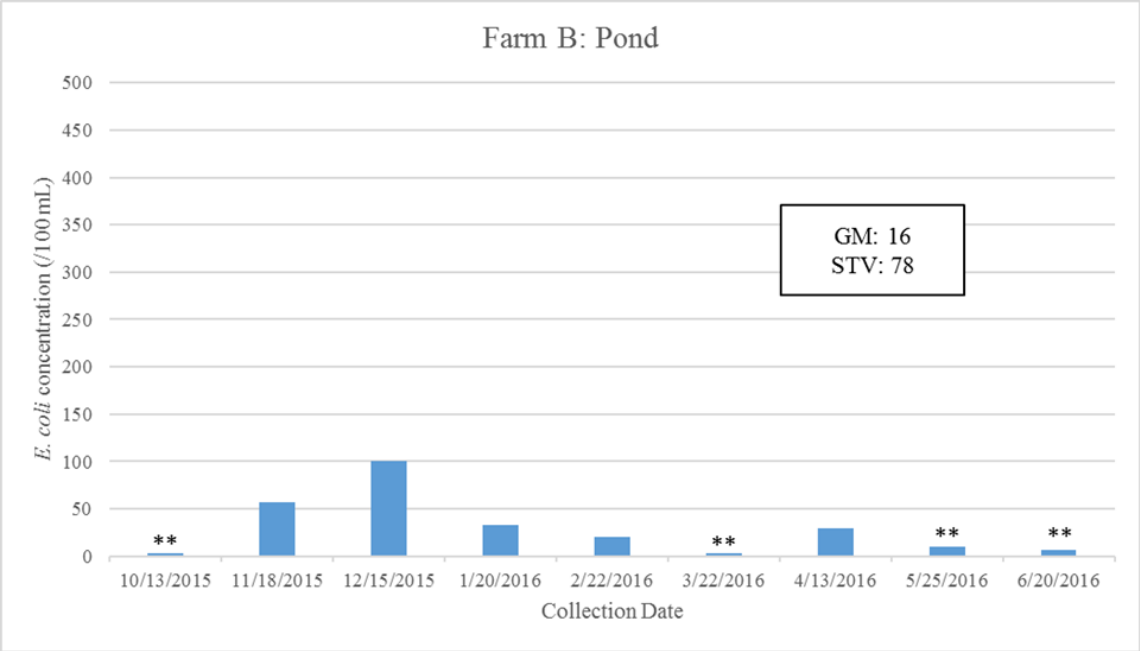

Figure 10: Generic *E. coli* concentrations in water samples collected from the pond on farm B.
** denotes *E. coli* concentrations below the detectible limit

3.3.3 Farm C:

Farm C is a 41-acre farm with 23 head of cattle, 11 horses, 30 to 40 goats, and 7 sheep. The sampled stream flows around the outer perimeter of this farm but is surrounded by grazed land on both sides (Figure 11). Cattle have free access to the pond, but the horses, goats, and sheep were held in separate, nearby pastures.

Four quarterly water samples were collected from the stream entrance and exit locations. Nine monthly samples were collected from the pond. USEPA method 1603 was used to enumerate generic E. coli/100 mL for each stream entrance and exit sample (Figure 12) and pond sample (Figure 13). Using these values, GM and STV were calculated for the stream entrance (Table 11), stream exit (Table 12), and pond (Table 13).

The stream entrance GM and STV results were 20 and 100 generic *E. coli*/100 mL, respectively. Stream exit GM and STV results were 90 and 832 generic *E. coli*/100 mL, respectively. GM and STV results for the pond samples were 95 and 516 generic *E. coli*/100 mL, respectively.

Based on these criteria, the stream entrance point does satisfy the Produce Safety Rule requirements for irrigation microbial water quality. However, the stream exit point and the pond do not meet the STV requirements, and therefore, would require a one-day die-off period for irrigation use.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 11: Farm C stream entrance (green star), stream exit (red octagon), and pond (yellow triangle) sampling sites.

Table 11: Farm C stream entrance GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	20	1.30	100	2.00
Deviation from criteria		-0.80		-0.61
Does your water meet PSR criteria?		Yes		Yes
Are corrective measures necessary?		No		No
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		0

Table 12: Farm C stream exit GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	90	1.96	832	2.92
Deviation from criteria		-0.14		0.31
Does your water meet PSR criteria?		Yes		No
Are corrective measures necessary?		No		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		1

Table 13: Farm C pond GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	95	1.98	516	2.71
Deviation from criteria		-0.12		0.10
Does your water meet PSR criteria?		Yes		No
Are corrective measures necessary?		No		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		1

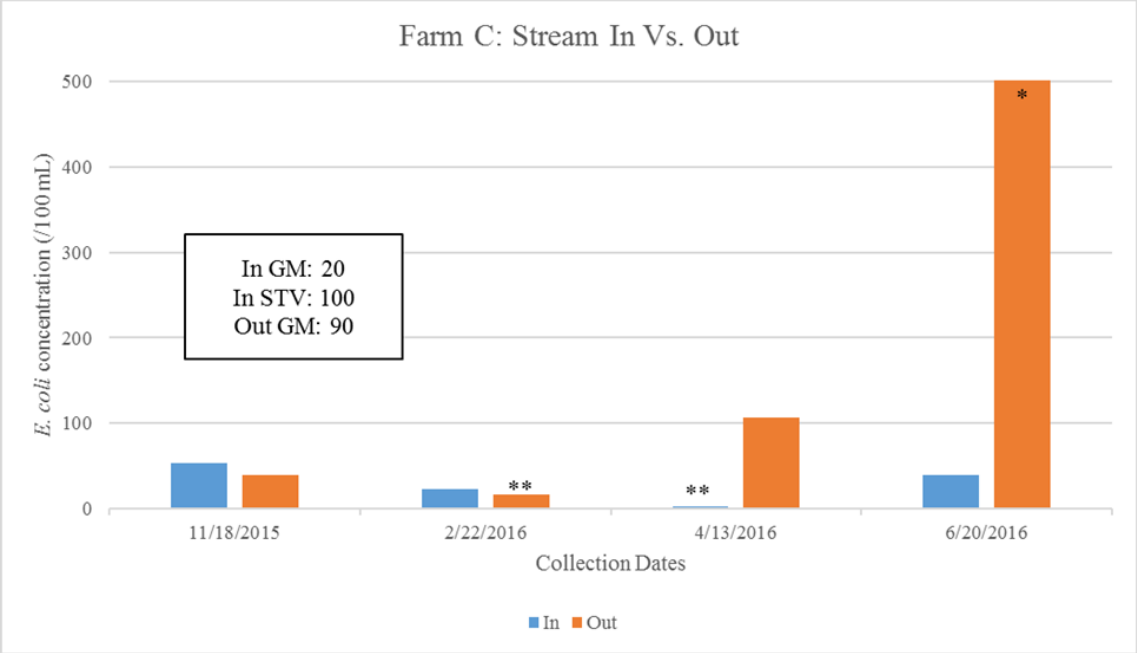

Figure 12: Generic *E. coli* concentrations in water samples collected from stream entrance and exit points on farm C.
* denotes *E. coli* concentrations greater than 500 CFU/100 mL
** denotes *E. coli* concentrations below the detectible limit

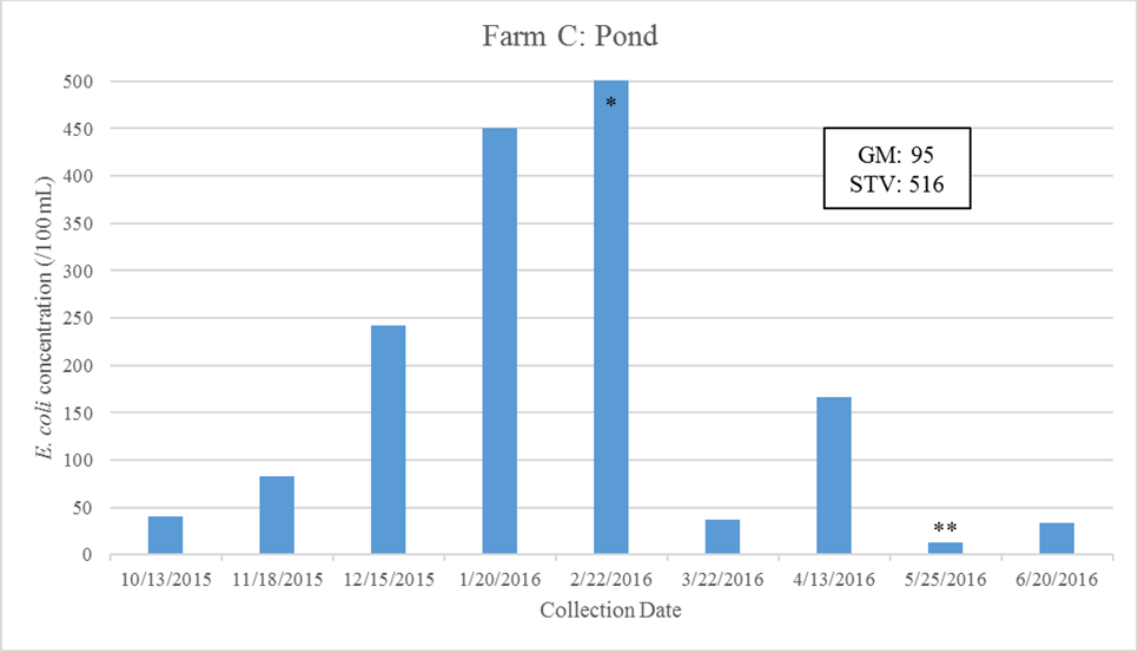

Figure 13: Generic *E. coli* concentrations in water samples collected from stream entrance and exit points on farm C.
* denotes *E. coli* concentrations greater than 500 CFU/100 mL
** denotes *E. coli* concentrations below the detectible limit

3.3.4 Farm D:

Farm D is a 60-acre farm housing approximately 15 head of cattle and 20 head of horses. This farm is dedicated to equine training, breeding, boarding, and sales, and is also a cow-calf operation. The sampled stream enters the farm near the equine portion, flows through two ponds, and exits on the opposite side of the cattle portion. Both the cattle and the horses have access to the water (Figure 14).

Four quarterly water samples were collected at the entrance and exit points of the stream; nine monthly water samples were collected from the pond. USEPA method 1603 was used to enumerate generic *E. coli*/100 mL for each entrance and exit samples (Figure 15) and each pond sample (Figure 16). Using these values, GM and STV were calculated for the stream entrance (Table 14), stream exit (Table 15), and pond (Table 16).

The stream entrance GM and STV results were 408 and 2205 generic *E. coli*/100 mL, respectively. Stream exit GM and STV results were 37 and 94 generic *E. coli*/100 mL, respectively. GM and STV results for the pond samples were 47 and 214 generic *E. coli*/100 mL, respectively.

Based on these criteria, the stream entrance point does not satisfy the Produce Safety Rule requirements for irrigation microbial water quality, and a two-day die-off period would be necessary to use this water for irrigation purposes. However, the stream exit point and the pond do meet the microbial water quality requirements, and therefore, could be used for irrigation purposes.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 14: Farm D stream entrance (green star), stream exit (red octagon), and pond (yellow triangle) sampling sites.

Table 14: Farm D stream entrance GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	408	2.61	2205	3.34
Deviation from criteria		0.51		0.73
Does your water meet PSR criteria?		No		No
Are corrective measures necessary?		Yes		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		2		2

Table 15: Farm D stream exit GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	37	1.56	94	1.97
Deviation from criteria		-0.54		-0.64
Does your water meet PSR criteria?		Yes		Yes
Are corrective measures necessary?		No		No
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		0

Table 16: Farm D pond GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	47	1.67	214	2.33
Deviation from criteria		-0.43		-0.28
Does your water meet PSR criteria?		Yes		Yes
Are corrective measures necessary?		No		No
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		0

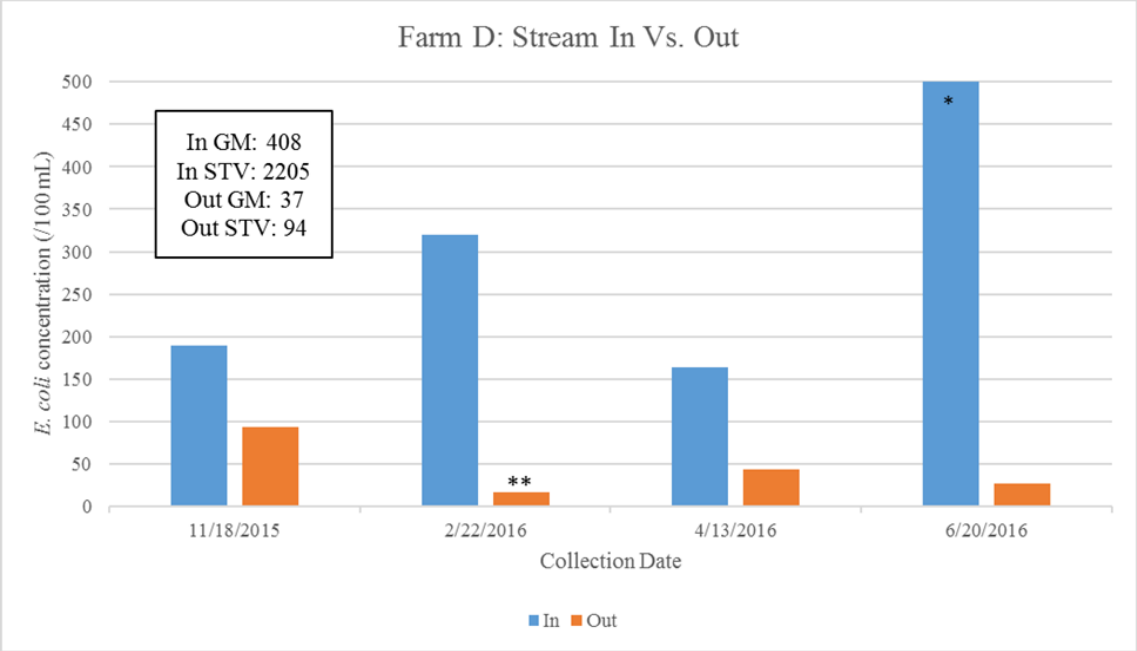

Figure 15: Generic *E. coli* concentrations in water samples collected from stream entrance and exit points on farm D.
*denotes *E. coli* concentrations greater than 500 CFU/100 mL
** denotes *E. coli* concentrations below the detectible limit

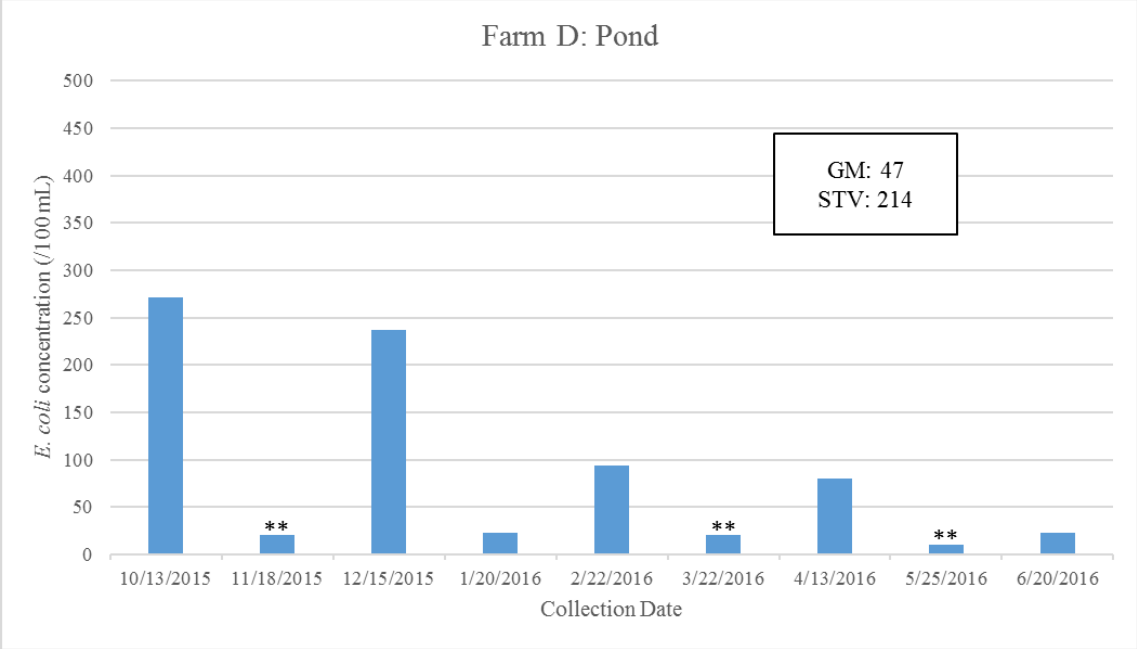

Figure 16: Generic E. coli concentrations in water samples collected from the pond on farm D.
*denotes E. coli concentrations greater than 500 CFU/100 mL.
** denotes E. coli concentrations below the detectible limit

3.3.5 Farm E:

Farm E is a 75-acre farm used for equine breeding, riding lessons, and hay sales, maintaining approximately 15 head year round. A stream was not available for sampling on this farm, but water samples were collected from the pond to which horse have free access (Figure 17). Nine monthly water samples were collected from the pond, and USEPA method 1603 was used to enumerate generic *E. coli*/100 mL in each sample (Figure 18). Using these values, GM and STV were calculated (Table 17).

The GM and STV values for the pond at farm E were calculated at 18 and 139 generic *E. coli*/100 mL respectively. These values meet the requirements outlined by the Produce Safety Rule for irrigation water, and no corrective action is necessary.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 17: Farm E pond (yellow triangle) sampling site

Table 17: Farm E pond GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	18	1.25	139	2.14
Deviation from criteria		-0.85		-0.47
Does your water meet PSR criteria?		Yes		Yes
Are corrective measures necessary?		No		No
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		0

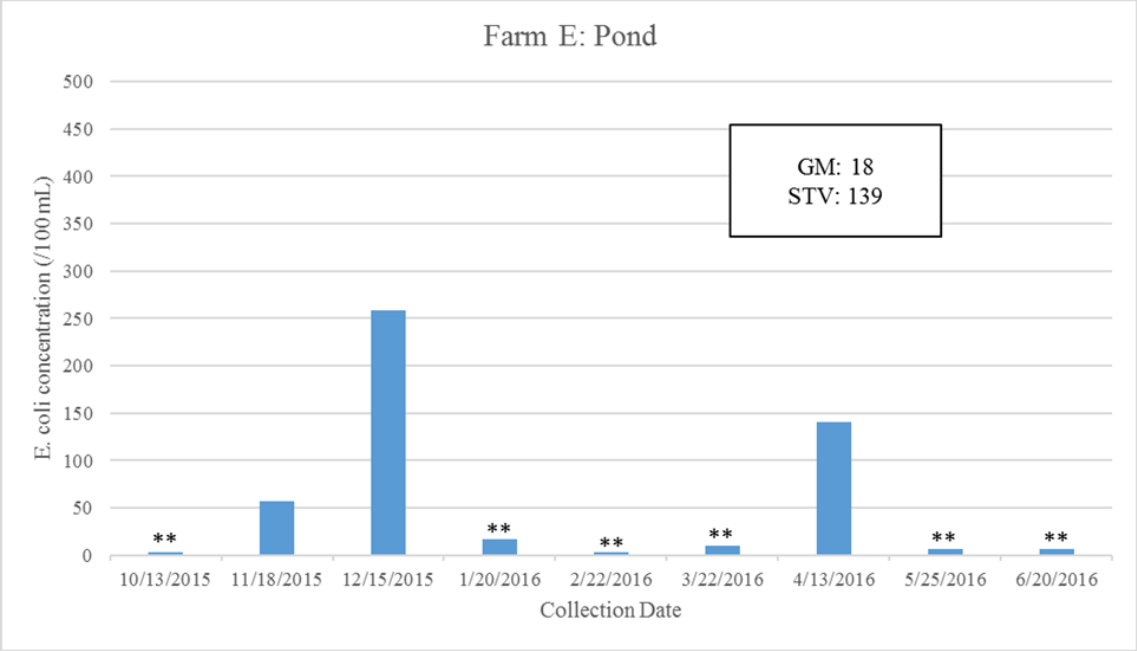

Figure 18: Generic *E. coli* concentrations in water samples collected from the pond on farm E.
** denotes *E. coli* concentrations below the detectible limit

3.3.6 Farm F:

Farm F is described as a 30-acre cow-calf operation housing approximately fifteen to twenty head of cattle. A stream was not available for sampling on this farm, but water samples were collected from the pond to which cattle have free access (Figure 19). Nine monthly samples were collected from the pond and USEPA method 1603 was used to enumerate generic *E. coli*/100 mL in each sample (Figure 20). Using these values, GM and STV were calculated (Table 18).

The GM and STV values for the pond at farm F were calculated at 93 and 476 generic *E. coli*/100 mL respectively. This pond source does not meet the requirements outlined by the Produce Safety Rule for irrigation water. The STV value exceeds the 410 generic *E. coli* CFU/100 mL limit. Therefore, a one-day die-off period would be necessary to use this water for crop irrigation.

For access to this figure, contact Luxin Wang Ph.D. at lzw0022@auburn.edu
Figure 19: Farm F pond (yellow triangle) sampling site

Table 18: Farm F pond GM, STV, and suggestion output from MWQP Excel tool.

	GM (Generic *E. coli* CFU/100 ml)	GM (Generic *E. coli* log CFU/100 ml)	STV (Generic *E. coli* CFU/100 ml)	STV (Generic *E. coli* log CFU/100 ml)
Produce Safety Rule Criteria	126	2.10	410	2.61
Your MWQP results	93	1.97	476	2.68
Deviation from criteria		-0.13		0.07
Does your water meet PSR criteria?		Yes		No
Are corrective measures necessary?		No		Yes
How many days are necessary if using microbial die-off between last irrigation and harvest? Apply the greater number of days based on GM or based on STV.		0		1

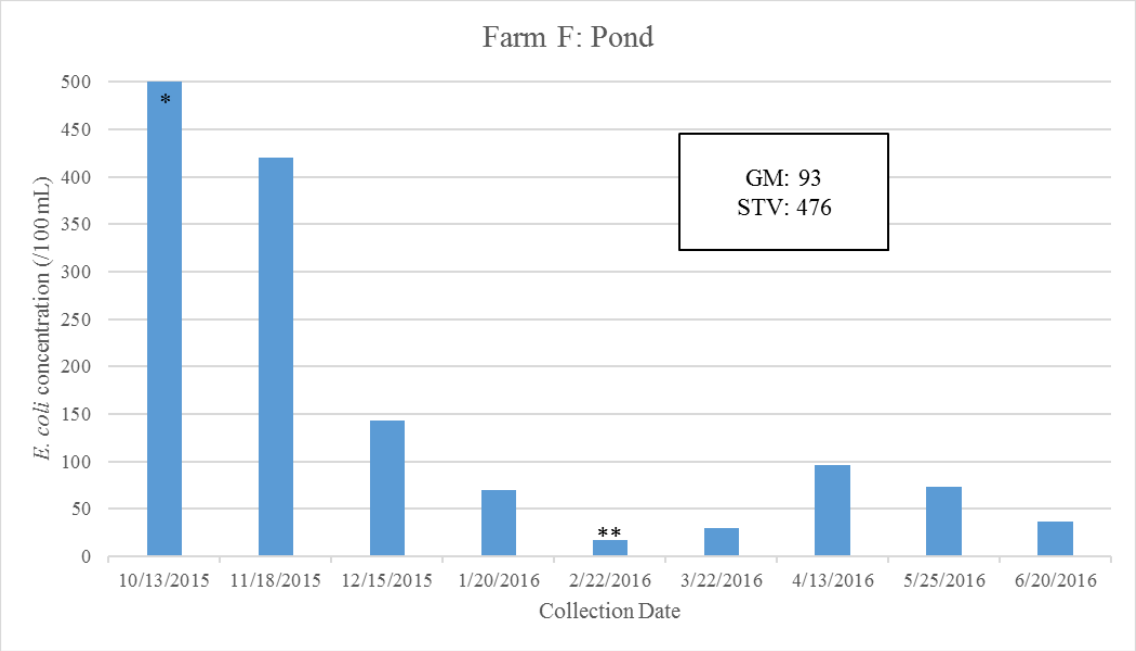

Figure 20: Generic *E. coli* concentrations in water samples collected from the pond on farm E.
*denotes *E. coli* concentrations greater than 500 CFU/100 mL
** denotes *E. coli* concentrations below the detectible limit

3.4 Discussion

With the implementation of the Produce Safety Rule resulting in more stringent federal regulations on agricultural water and crop production, producers are facing uncertainty about their water supplies. Surface water used for irrigation purposes typically comes from streams, ponds, lakes, and other reservoirs. Microbial evaluation of water sources from farms with varying degrees of animal agriculture may help producers better understand the effects the Produce Safety Rule will have on surface water production. Each of the farms selected for this survey study represents a different scenario that producers may face.

For each of the streams, quarterly surface water samples were collected at the entrance and exit points over the course of one year to represent each of the seasons. For each of the ponds, surface water samples were collected monthly over the course of nine months. The Produce Safety Rule requires that twenty samples be collected and analyzed over the course of two to four years to build an initial data base for each water source. Given the samples collected over this short period of time, the pond located on farm B, stream entrance on farm C, stream exit and pond on farm D, and the pond on farm E are all currently trending toward meeting the microbial requirements outlined by the Produce Safety Rule. As more water samples are collected, it is possible that this may change. While the other sampling locations do not currently meet the requirements, it is likely that may change once more samples have been analyzed.

Animal agriculture does not seem to always have a negative impact on the microbial quality of surface water. This is evident when evaluating streams such as the

one found on farm D. According to the collected samples, the entrance point of this stream does not meet the GM or STV values outlined by the Produce Safety Rule. However, with the samples provided, the pond and exit points of this stream do meet the microbial water standards. This is why it is important to monitor the microbial quality of each water source.

The Produce Safety Alliance at Cornell University has developed an easy to use Excel tool for farmers to monitor their water quality. The input of generic *E. coli* counts will generate an output file that will tell producers if their water meets the standards. For farmers whose water source does not meet the microbial water quality standards for the Produce Safety Rule, this Excel tool will inform the producer if a die-off period will be effective and how long the die-off period must be. Producers may choose to follow this guideline or implement other measures such as physical or chemical treatment of the water.

3.5 References

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Chapter 4: Conclusions

With the continuing threat of foodborne illness in the United States, it has become increasingly important to develop and implement preventative strategies to protect our food supply. Historically, foodborne pathogens such as *Salmonella* spp. and pathogenic *Escherichia coli* have been closely associated with food products of animal origin. In the past few decades, outbreaks associated with crops have become a great concern. The contamination of soil and water leading to contamination of crops has become the focus of much discussion in recent years. Because many crops are consumed in their raw state, determining points of contamination, utilizing good agricultural practices, and developing scientific based preventative measures are important in protecting these food commodities.

In the first portion of this study, the transfer and persistence of *Salmonella* spp. in an animal production environment, we demonstrated that *Salmonella* spp. originating from poultry houses has been transferred in the environment. These *Salmonella* spp. strains reached not only the immediate spots around the poultry farm but go beyond the defined boundaries. Rainfall and other environmental factors, wild animals, farm equipment, and workers can contribute to the spread of these pathogens from one source to other areas of the environment and even nearby surface water supplies. Once in the environment or the water supply, these pathogens have the potential to survive for long periods of time and cross contaminate other animal species or food commodities.

The second portion of this study, evaluation of surface water quality on animal agriculture farms, focused on evaluating the effects FDA regulations could have on

surface water sources and crop producers. With the implementation of the Produce Safety Rule, the microbial quality of surface water sources to be used for crop irrigation will now be monitored and regulated by the FDA. The effects of these new regulations had not yet been evaluated. By evaluating the generic *E. coli* concentration in surface water sources at several points on farms around East Central Alabama, we were able to accomplish two goals: evaluate the impact of animal agriculture on the surface water quality at these particular farms, and demonstrate how the Produce Safety Rule is used and interpret by utilizing the Cornell University Excel tool.

It was determined that alone, animal agriculture does not always have a negative impact on the surface water quality. It seems that other factors may have an influence on the water quality such as the type and scale of animal agriculture, geographical layout of the land, rainfall events, temperature, and other weather events.

It was also determined that use of the Excel tool developed by Cornell University is an efficient method of monitoring surface water quality. By using this tool to calculate GM and STV, producers will have confidence in their water supplies. While some water supplies may not meet the standards outlined by the Produce Safety Rule, there are preventative measures such as die-off periods and physical and chemical treatments, that can be implemented to safeguard their crops.