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Filth Flies as a Vector for Some Pathogenic Bacteria Transfer

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FILTH FLIES AS A VECTOR FOR SOME PATHOGENIC BACTERIA TRANSFER

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ABSTRACT

Two separate sets of experiments were conducted to determine the transfer of bacteria by flies. An *Escherichia coli* ampicillin-resistant strain with a fluorescent gene was used during the experiments. The first set of experiments were divided into two trials to measure the transfer of *E. coli* by fruit flies to apple slices and bologna during short term exposure. Short time exposure (1, 5 and 15 min) of flies to inoculated apple slices were tested in the first trial to determine the transfer of *E. coli* to flies. No difference ($P>0.05$) in the number of bacteria transferred to flies were found associated with these exposure times. In the second trial, the transfer of *E. coli* from inoculated apple or bologna slices (5 min exposure) to un-inoculated slices (1, 5 and 15 min exposure) were evaluated. More bacteria were transferred to bologna after 1 and 5 min exposure times compared to transfer to apples, while the number of cells transferred did not differ for bologna and apples after 15 min exposure. The percentage of *E. coli* transferred from inoculated food to flies was low (<0.5%) while the percentage transferred from flies to un-inoculated food was relatively high (>50%). This study found that flies can pick up and transfer bacteria to food in short exposure times.

Filth flies, especially house flies, can harbor and ultimately distribute human pathogens to food and food contact surfaces. To determine the potential of flying insects collected from poultry growout houses to carry *Salmonella* and *Campylobacter*, a total of 2164 flies were caught on poultry farms located in the Upstate, Middle, and Coastal regions of South Carolina and segregated based on fly family type. Capture flying insects included house flies in the family Muscidae inside the poultry house [in-HF] (N = 289), house flies just outside the poultry house [out-HF] (N = 1023), and house flies 100 meters from the poultry houses [100m-HF] (N = 547). Other flying insects included wasps in the family Vespidae species (spp.) captured just outside the poultry house

[out-Vespidae] (N = 71), Vespids spp. 100 meters from the poultry house [100m-Vespids] (N = 126), flesh flies in the family Sarcophagidae just outside the poultry house [out-Sarcophagids] (N = 13), and flesh flies 100 meters from the poultry house [100m-Sarcophagids] (N = 9), blow flies in the family Calliphoridae 100 meters from the poultry house (100m-Calliphorids), darkling beetles in the family Tenebrionidae just outside the poultry house [out-DB] (N = 30), and darkling beetles 100 meters from the poultry house [100m-DB] (N = 56). Populations of *Campylobacter spp., Salmonella spp.,* and total aerobic microorganisms (APC) were recovered from the flies as well as the number of *Salmonella spp.* and *Campylobacter spp*. positive flies at a 100 m distance from the farms. Along with fly groups, chicken feces in the houses [CF] from three farms, cow manure around farm 1 and farm 2, and dog feces around farm 1 were also sampled. While no *Campylobacter jejuni* was detected from any of the samples, including fly groups, chicken feces, cow manures, and dog feces, *Campylobacter coli* positive samples were detected in the cow manure samples in both replications, 100m-Calliphoridae, out-HF and 100m-DB in one out two replications on farm 2. Moreover, positive Serogroup B *Salmonella spp.* were determined in the groups in-CF, in-HF, and out-HF on farm 2 and positive Serogroup C *Salmonella spp.* were determined in the groups of in-CF, out-HF, and 100m-HF on farm 3. Data demonstrates that house flies may be a vector in the transmission of *Salmonella spp.* from poultry farms.

DEDICATION

I would like to dedicate this work to my family. Without all your support, this hard work would not have been possible.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

The Centers for Disease Control (CDC) estimates that there are 48 million cases of foodborne illness, 128,000 hospitalizations, and 3,000 deaths annually in the U.S. (CDC, 2022a). The top pathogens that cause foodborne illness in the U.S. are *Norovirus* (58%), nontyphoidal *Salmonella spp.* (11%), *Clostridium Perfringens* (10%), *Campylobacter spp.* (9%). The top pathogens leading to hospitalization are *Salmonella* spp. (28%), *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%) and *Norovirus* (11%) (Scallan et al., 2011). Additional discussion on *Salmonella* and *Campylobacter* is provided below. The CDC estimates that *Salmonella* causes approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths each year (CDC, 2022c). *Campylobacter* infections result in 1.5 million illnesses each year in the United States (CDC, 2022b). *Campylobacter* and *Salmonella* are the two primary pathogens causing human gastroenteritis related to poultry meat consumption (Rouger et al., 2017)**.** Based on the samples randomly collected from retail stores in Iran, the highest prevalence of *Campylobacter spp.* was determined in chicken (47.0%), followed by quail (43.0%), partridge (35.3%), turkey (28.8%), and ostrich (4.8%) meat (Rahimi & Ameri, 2011). According to the 2018 The European Food Safety Authority (EFSA) report, the highest prevalence of *Campylobacter spp.* was in chicken meat (37.5%) and turkey meat (28.2%) (EFSA, 2019).

Salmonella species

Salmonella is a genus of a gram-negative, motile, non-spore-forming, rod-shaped bacterium in the Enterobacteriaceae family. *S. enterica* and *S. bongori* are the two species of *Salmonella* that can cause illness in humans. *Salmonella enterica* is the most significant health concern and comprises six subspecies: (1) *S. enterica* subsp. *enterica*, (2) *S. enterica* subsp. *salamae*, (3) *S. enterica* subsp. *arizo*nae, (4) *S. enterica* subsp. *diarizonae*, (5) *S. enterica* subsp. *houtenae*, and (6) S. enterica subsp. indica (FDA Bad Bug Book, 2012). *Salmonella* spp. are further subdivided into approximately 2600 serotypes. S. *enteritidis* and S. *typhimurium* are ubiquitous in the US, and those are just two of the numerous serotypes of the *Salmonella enterica* subsp. *enterica* species. Depending on the serotypes, *Salmonella* can cause two types of illness. 1. Nontyphoidal salmonellosis (NTS) is caused by serotypes other than *S. typhi* and *S. paratyphi* A. and leads to nausea, vomiting, diarrhea, fever, cramps, and headache. 2. Typhoid Fever is caused by serotypes *S. typhi* and *S. paratyphi* A, found only in humans. It leads to the symptoms of high fever, headache, lethargy, abdominal pains and diarrhea, and loss of appetite. Typhoid fever is more severe than NTS, with a 10% mortality rate, if untreated after infection (FDA Bad Bug Book, 2012). *Salmonella typhimurium, S. enteritidis*, and *S. newport* are the most common serotypes in food products and are responsible for 50% of salmonellosis (Porwollik et al., 2004). Based on the FoodNet 2021 report, the five most common *Salmonella* serotypes are *enteritidis, newport, typhimurium, javiana*, and i 4,[5],12:i:- since 2010 (Collins et al., 2022).

Source of *Salmonella* infection

Salmonella can live in the gastrointestinal tracts of livestock, wildlife, domestic pets, and humans (FDA Bad Bug Book, 2012). *Salmonella* can infect people through consumption of eggs, meat,

dairy products, vegetables, and water (Popa & Ioan Popa, 2021). Animal sources cause about 46.4% of human *Salmonella* infections, and poultry creates the majority of these (Sanchez et al., 2002). Environmental sources, such as rodents, wild birds, feed, insects, transportation, and farm environment, may cause a *Salmonella* outbreak in a poultry flock (Bailey et al., 2001). However, poultry feed may also be a source of infection (Jarquin et al., 2009). Jajere et al., (2019) reported that *Salmonella* was detected in 5.14% (9/175) of drinking water, 7.14% (5/70) of poultry feed, and 5.0% (3/60) from fly samples collected from 35 chicken flocks in Malaysia. The hatchery might be the most important source of *Salmonella* contamination in a poultry operation among other sources for two reasons. First, newly hatched chicks are more sensitive to *Salmonella* colonization due to an absence of protective gut microflora. Second, chicks in the hatchery are often exposed to *Salmonella* (Bailey et al., 2001), which can be caused by particles like dander, feathers, and fluff in the air or aerosols while they are waiting in chick trays. Moreover, less than five cells of *Salmonella* could be enough to colonize one-day-old chicks, but infection in older birds is irregular and requires higher doses (Bailey et al., 2001; Milner & Shaffer, 1952). Cox et al. (1990) confirmed that only two cells of *Salmonella* caused colonization of the young chicks. However, two-week-old chicks were more resistant to intestinal colonization by *Salmonella* (Bailey et al., 2001; Barnes et al., 1972). *Salmonella* serovar *enteritidis* persisted for at least two weeks in a fly host after *Salmonella* contamination (Holt et al., 2007)**.** The entire flock can be infected within 2 to 10 days once a bird in the flock is infected by *Salmonella* (Jarquin et al., 2009). *Salmonella* infections in poultry can be both vertical and horizontal transmission (Dar et al., 2017). With an infectious does being 10 - 10^7 CFU/ml (Lukinmaa, 2003).

Campylobacter

Campylobacter is a gram-negative rod, curved to S-shaped, non-spore-forming bacteria. Many strains have motility due to the flagella attached at one or both polar ends of the bacteria. *Campylobacter* species are generally fragile in the ambient air because they grow at lower oxygen concentrations (microaerophilic). They are also sensitive to drying, heating, freezing, disinfectants, and acidic conditions. Twenty-five species of the genus *Campylobacter* are significant human pathogens, including *Campylobacter jejuni, Campylobacter coli, Campylobacter fetus, Campylobacter lari, Campylobacter upsaliensis,* and *Campylobacter hyointestinalis* (Fontanot et al., 2014; Lynch et al., 2011). *Campylobacter jejuni, Campylobacter coli, and Campylobacter lari* are the most common species causing human infection. They can be isolated from poultry and are the most significant concern in the poultry industry (Fontanot et al., 2014). *Campylobacter jejuni* causes 90% of camplyobacteriosis, followed by *Campylobacter coli* (2.5%) and *Campylobacter lari* (0.20%) (Saiyudthong et al., 2015). The primary symptoms of campylobacteriosis are diarrhea (sometimes bloody), fever, vomiting, and abdominal cramps (FDA Bad Bug Book, 2012).

Source of *Campylobacter* infection

Most food-producing animals, such as chickens, turkeys, swine, cattle, and sheep, may have *Campylobacter* a part of the natural gut microflora (FDA Bad Bug Book, 2012). Although many poultry species, mainly commercial chickens and turkeys, generally carry *Campylobacter* in their intestines without any sign of disease, *Campylobacter* is the main reason for food-borne gastroenteritis in people worldwide. Poultry meat, raw milk, and contaminated water are the most common sources of *Campylobacter* outbreaks (Sahin et al., 2015).

Horizontal transmission from environmental sources was shown as the main route of *Campylobacter* in a poultry house (Agunos et al., 2014; Hermans et al., 2012; Newell & Fearnley, 2003; Sahin et al., 2002). Environmental sources or factors could be flies, rodents, wild birds, poor biosecurity on the farm, ventilators, seasons, farm workers, domestic pets, use of old litter, number of houses on a farm, slaughter age, and size of flocks. Feed, water, and fresh litter are not prevalent sources for the first introduction of *Campylobacter* in the house (Sahin et al., 2015). Commercial poultry flocks are often initially *Campylobacter*-free and usually remain that way until 2–3 weeks of age (Allen et al., 2011; Newell & Fearnley, 2003; Patriarchi et al., 2011; Ridley et al., 2011; Sahin et al., 2015). Maternal antibodies found extensively in young chicks are shown to partially protect young chicks against *Campylobacter* colonization (Cawthraw & Newell, 2010; Sahin, Luo, et al., 2003). Regardless of species of poultry (turkey and chicken) and production types (conventional and free range/organic), *Campylobacter* is rarely detected in young birds less than 2–3 weeks of age (Sahin et al., 2015). No *Campylobacter* was found during the first three weeks of age in multiple broiler flocks under commercial houses despite low biosecurity measures such as a high fly population, poor hygiene conditions, presence of layer birds, multi-age broiler flocks (Kalupahana et al., 2013; Sahin et al., 2015). Once contamination occurs, most birds are infected quickly, and the overall prevalence in the house is usually nearly 100% at the slaughter age (Bemdtson et al., 1996; Cardinale et al., 2004; Goddard et al., 2014). Instead of vertical transmission via eggs, horizontal transmission from the environment is the primary source of *Campylobacter* colonization in poultry (Barrios et al., 2006; Callicott et al., 2006; Sahin, Kobalka, et al., 2003). Birds eventually become colonized by *Campylobacter* with age and reach the maximum level (mainly close to %100) at the slaughter age (Barrios et al., 2006; Cardinale et al., 2004; Sahin et al., 2015). However, this is seasonal, with lower prevalence in the winter months

and when it is very dry. In a Danish study, *Campylobacter spp.* in individual chickens and flocks varied depending on the season in one year. The percentage of positive cases was higher during the late summer and autumn months (July-October), with the highest rates (78-80%) observed in August. However, during the winter months (December-March), the percentage of positive cases was lower overall, with the lowest rates (13-20%) occurring in March ((Bang et al., 2003). According to FDA bad Bug Book, 2012, "typically, a poultry carcass can carry 100 to 100,000 *Campylobacter* cells after contamination". However, this is misleading. For example, Messens, (2015) reported more than 50% of poultry carcasses had less than 100 CFU/g, and only 5.8% had more than 10,000 CFU/g. Mishandling or undercooking poultry products has a substantial risk for humans since only 500 *Campylobacter* cells can cause infection (FDA Bad Bug Book, 2012).

House Flies (*Musca Domestica*)

Adult house flies are 3–8 mm long and deposit eggs on or just under the surface of moist substrates. House fly eggs are about 1 mm long and creamy white in color. House flies are located chiefly at animal production facilities but also appear in the urban environment. The larvae develop in decaying vegetable materials, feces, and household garbage (Geden et al., 2021). The entire lifecycle of a housefly (egg to emerged adult) transpires in 8 days under optimal conditions (Hevitt, 1908), but it is normally completed in 10-21 days (Nayduch & Burrus, 2017). Due to the sponging mouthparts, adult house flies must consume either liquid food or exude a droplet of regurgitate (called bubbling) onto the solid food to soften them before ingestion (Geden et al., 2021). It is thought that bubbling concentrates the nutrients in the ingested food and decreases the weight the fly must carry during the flight by eliminating excess water (Hendrichs et al., 1992; Stoffolano, 2019). According to Gomes et al. (2018), bubbling also plays a thermoregulation role in lowering

body temperature by employing evaporative cooling. House flies with enough nutrients under laboratory conditions can lay 100–150 eggs per female. An individual female can lay 900 in her lifetime because flies can produce up to six batches of eggs with adequate food and conditions (West, 1951). However, egg deposition and fly longevity are much lower under field conditions since food resources are usually limited. An adult female housefly can live up to 6 weeks under optimal laboratory conditions, however, it is estimated that flies survive 1-6 days on a poultry farm and 3-19 days on dairy farms (Geden et al., 2021).

Larval house flies can make a difference on pathogen destruction in the manure

There is a relationship between all stages of houseflies and bacteria. Adult houseflies are metropolitan, sociable, and synanthropic, a potential reservoir and mechanical or biological transmitter of pathogens, so they threaten public and animal health. Larval houseflies depend on bacteria as food and stay close to their developmental site. Bacteria digestion by larvae can be crucial in decomposing and recycling nutrients in the food chain. Pathogen destruction in manure can provide better waste management, making it an excellent fertilizer for food crops (Nayduch & Burrus, 2017). Zhu et al. (2015) inoculated housefly larvae into pig manure for composting instead of using bulking agents. They concluded that this technology could produce high-quality organic fertilizer without bulking agents, which are added to pig manure to decrease moisture. Larvae can be harvested from composted manure and used as food for fish. Not using bulking agents in the manure saves costs on the farm and retains N, P, and K content in organic fertilizers undiluted (Zhu et al., 2015).

Transmission of disease by flies

The presence of filth flies, especially house flies, has attracted great attention in transmitting human pathogens. Forty-seven species of flies categorized as "filth flies" might disseminate foodborne pathogens. Twenty-one of 47 species are a potential threat to human health and classified as "disease-causing flies" based on these scientific criteria: Association with *E. coli, Salmonella*, and *Shigella*; Association with humans (synanthropic); 1) Association with *E. coli, Salmonella,* and *Shigella*; 2) Association with humans (synanthropic); 3) Association with the domestic environment (endophilic); 4) Communicative act (quickly integrating into human populated areas from unsanitary conditions); 5) Attraction to both excrement and human food products; and 6) Recognition by authorities as a potential health hazard (Butler et al., 2010; Olsen, 1998). The synanthropic house fly, *Musca domestica (Diptera: Muscidae),* is a potential reservoir and transmitter for a diverse range of pathogens (bacteria, fungi, viruses, and parasites), some of which lead to intensive diseases in humans and animals, infact more than 130 pathogens were isolated from the house fly (Khamesipour et al., 2018).

Houseflies may transfer the pathogens by the sponging mouthparts and on the body surface and leg hairs, on the sticky parts of the feet, regurgitation of vomitus, and the alimentary tract (Olsen, 1998; Rosef & Kapperud, 1983; West, 1951). One study demonstrated that *C. jejuni* between chicken flocks was transferred by flies, and *Campylobacter* could be recovered up to 20% from the feet and ventral surface of the body and 70% from the viscera (Shane et al., 1985). Holt et al. (2007) showed that *Salmonella* was recovered from all fly intestinal tracks (100%), while only 15% of the crops and none of the salivary gland. It indicates that defecation may play a more prominent role in *Salmonella* spread than crop regurgitation by the flies. Similar results were observed in the other study, with 89% for gut and 11% for crop samples (Greenberg et al., 1970).

One research reported that using a phosphate-buffered saline (PBS) solution incorporated with 0.5% detergent (Tween 20) significantly increased *Salmonella* recovery from the exterior part of the fly, which indicates that *Salmonella* is not simply sitting on the surface of the fly. However, there is a more active interaction between *Salmonella* and the exterior part of the fly. Additionally, other mechanisms may be more effective at transferring bacteria to different surfaces rather than simple physical contact (Holt et al., 2007). Although most flies remain on or near where they emerge, they can travel relatively long distances (Geden et al., 2021), in fact flies have been reported to travel more than 12 km from where they emerged (Bishopp & Laake, 1921; Geden et al., 2021; Quarterman et al., 1954). Yates et al., (1952) caught house flies 0.5, 1, 8 and even 20 miles from the release point. A review paper by Khamesipour et al., (2018) examined 99 selected research titles between 1970–2017 from 21 countries regarding human pathogens carried by the housefly based on the following criteria: Isolated pathogen types and species (bacteria, fungi, viruses, and parasites), housefly stage (might be larva or adults or both), study type (might be experimental or field), house fly site from where pathogen isolated (might be surface or gut or both), nature of pathogens (whether carrying antimicrobial-resistant genes or not), house fly capturing locations (farms, human residents, markets/shops, hospitals). Among these 99 titles, 69 studies described bacterial pathogens, 15 fungi, three bacteria & fungi, four parasites, one parasite & bacteria, and seven viral. More than 68% (68.69%) of the studies were field studies (performed on house flies caught in the wild), while 31 (31.31%) were experimental studies (conducted in the laboratory). Twenty studies addressed house flies captured from within human habitation and twenty-eight from animal farms (poultry, dairy, and swine farms). The rest are from the surroundings, food centers (including cafeterias and restaurants), markets, hospitals, dump sites or sanitary landfills, and gardens/farms. Forty-four studies indicated that pathogens were isolated

from the body surfaces of the flies, 33 studies showed the isolation from both the body surfaces and the gut, and 22 studies reported the pathogen isolation from the gut (Khamesipour et al., 2018).

House flies transmit bacterial pathogens from the contaminated locations

Rosef & Kapperud (1983) found that house flies might play a role in *Campylobacter* infection in humans by transferring bacteria from animals to human food. They found that 147 of 518 house flies from four different locations, including one cattle barn (100 flies), one turkey farm (103 flies), one swine (169 flies), and one chicken farm (146 flies), carried *Campylobacter*. While no strains were isolated from the cattle barn and turkey farm, 50.7% of positive *Campylobacter* flies were captured on the chicken farm and 43.2% in flies from a swine farm. The percent recoveries were determined as *Campylobacter coli* (90.1%), *C. jejuni* (6.2%), and nalidixic acid-resistant thermophilic *Campylobacters* (3.7%). Alam & Zurek (2004) supported the premise that house flies in cattle farms play a role in disseminating *E. coli* O157:H7 among animals and the surrounding environment. In this study, E. coli O157:H7 counts ranged from 3.0 X 10^1 to 1.5 X 10^5 CFU/fly. Greenberg et al. (1963) previously reported that house flies transferred *Salmonella* from slaughterhouses to nearby markets and residential areas in Mexico. In another study (Ommi et al., 2017), the carriage of *Campylobacter* and *Salmonella* from house flies was investigated in kitchens, animal farms, hospitals, and slaughterhouses in two provinces of Iran between June 2013 and May 2014. The overall detection of *Salmonella* and *Campylobacter* from houseflies was 15.8 % (95/600) and 19.5 % (117/600), respectively. Although a similar recovery frequency was observed in different sampling locations, cattle farms, animal hospitals, and slaughterhouses indicated significantly higher total recovery frequencies than kitchens, chicken farms, and human hospitals (P\0.05) (Ommi et al., 2017). Choo et al. (2011) also reported that house flies

carried *Campylobacter* and *Salmonella*. In this study a total of 60 house flies were captured in three locations (20 flies from each) of an animal teaching site where on a university campus, two cafeterias in the university where one is about 100 m, and the other 300 m away from the facility, and a poultry farm was about 30 km from the campus. They found that five percent (5%) and 13.3% of flies sampled were positive for *Campylobacter* and *Salmonella*, respectively. Two *Campylobacter coli* and one *Campylobacter jejuni* isolate were identified from the poultry farm. In contrast, no positive *Campylobacter* fly samples were detected from the animal facility and cafeteria. Two positive *Campylobacter* samples were from the external body surface, and one was from the internal contents of the flies. A total of eight samples were *Salmonella* positive from three locations. Another example of farm to food transfer was demonstrated when a three-year-old boy in a nursery school in Japan was taken to the hospital with bloody diarrhea caused by a verotoxin-producing *E. coli* (VTEC) O157:H7. To investigate the possible sources of VTEC O157:H7 infection, they tested samples of feces from fowl kept in the nursery school, the food, water from the cleaner, the sand pit, drainage effluent, and other suspected places in the nursery school. VTEC O157:H7 was not isolated from these samples. After some investigations, it was demonstrated that the children became infected in the dining room via food, plates, and utensils contaminated by house flies from cattle farms, the nearest located about 30 m from the school (Moriya et al., 1999). One study in India showed that houseflies act as mechanical vectors of *Vibrio cholerae* and may help their dissemination and make people sick. One hundred and fifty houseflies were captured from animal pens, yards, and houses (kitchen and sifting rooms) where a cholera outbreak was encountered. The flies were pooled into ten groups of 15 flies each. Of the ten fly pools examined, six (60%) were positive for *V. cholerae*. It was demonstrated that three of these

six pools of flies had the same species of *V. Cholerae*, isolated from the stools of patients suffering from diarrhea (Fotedar, 2001).

House flies as a source of bacterial infection from outside sources to farm animals

A Danish field study conducted in 2004 showed that flies had a potential threat of *Campylobacter* infection from outside animals to the broiler flocks through the ventilation system. They demonstrated that hundreds of flies passed through the ventilation system into the house daily in Summer. Approximately 8% of (4/49) flies captured in the house were culture positive, while 70.2% of (33/47) flies were PCR-positive for *Campylobacter* (Hald et al., 2004).

House flies as antimicrobial resistance pathogen carriers

Antimicrobial resistance of *Salmonella spp.* and *Campylobacter spp.* isolates against 20 antimicrobial agents were tested and it was determined that these bacteria resist the antimicrobials at low to moderate levels (Ommi et al., 2017). It was concluded that houseflies be considered an essential vector of antimicrobial-resistant pathogens, including *Campylobacter* and *Salmonella*. In another study, 3.7% of 147 *Campylobacter*-positive house flies collected from chicken and swine farms were nalidixic acid-resistant thermophilic *Campylobacters* (Rosef & Kapperud, 1983). Xu et al. (2018) reported that of 1650 flies captured from 33 cattle farms in the US, 11% of the flies (185) tested positive for *Salmonella* and 28% of positive isolates were resistant to at least three antibiotics (multidrug-resistant). Macovei et al. (2008) showed that house flies from a cattle feedlot could contaminate ready-to-eat food and drinks with multidrug-resistant *Enterococci*. House flies captured in a cattle feedlot exposed to a beef patty transferred *Enterococci faecalis* isolates which were resistant to several antibiotics, consisting of ciprofloxacin (17.4% of isolates), tetracycline

(13.0%), erythromycin (13.0%), and chloramphenicol (4.3%). In other research (Wang et al., 2011), 144 *Salmonella* isolates, 58 (26.4%) flies, and 86 (19.5%) swine stool were recovered from house flies and swine fecal samples from 11farms in two counties in Taiwan. 71.5% of the positive isolates were determined as multidrug-resistant. Furthermore, a high prevalence of multidrug resistance *Salmonella spp.* and *Shigella* isolates of 94% and 87% resistance were detected in flies caught at cattle farms, barbeque, and in urban settings in Australia (Vriesekoop & Shaw, 2010). Thirty-one of 34 house flies (91.2%) captured at a fish market carried multi-drug resistant *Salmonella spp.* (Sobur et al., 2019).

Research regarding the fate of the bacteria post ingestion by house flies

A lab-based study showed that the ingested *E. coli* O157:H7 by 6–8-day old house flies was reserved in the intestine and viable in the excreta of the flies at least three days after feeding. A large number of *E. coli* O157:H7 survived on the surface of the housefly mouthparts, and the flies were able to spread *E. coli* O157:H7 from the mouthparts for at least three days after feeding (Kobayashi et al., 1999). Another study reported that *C. jejuni* remained in the adult house fly's vomitus and fecal for 4 hours, but no viable bacteria were detected ≥ 8 h (Gill et al., 2017). In the other research, *S. aureus* persisted up to 6 h post ingestion in house flies but significantly decreased (Nayduch et al., 2013). However, *E. coli O157:H7* (Sasaki et al., 2000) and *Pseudomonas aeruginosa* (Joyner et al., 2013) multiplied within the house fly. Bahrndorff et al. (2014) demonstrated that *Campylobacter jejuni* was transmitted from the larvae stage to the pupae stage of *M. domestica* but not to the adult stage. The number of *C. jejuni* did not increase within larvae or pupae of the house fly after the bacterial exposure, but it declined during pupal development.

Potential of house flies to contaminate food with bacteria or from food to other substances

Macovei et al. (2008) also assessed the quantitative and qualitative contamination of ready-to-eat food with *Enterococci* from a natural population of house flies. A group of flies (5, 10, 20, and 40) was exposed to a beef patty, from a ready-to-eat hamburger, with a different exposure time (0.5, 1, 3, and 24 h). Irrespective of the number of house flies and the exposure time, all beef samples were contaminated with *Enterococci*. They found that the contamination rate of *Enterococci* in ready-to-eat food increased with the increasing number of houseflies and the increasing time exposure. 0.5 h exposure time ended with the contamination ranging from 3.1 X 10^3 CFU/g (5 house flies) to 8.4 X 10^4 CFU/g (40 house flies). De Jesús et al. (2004) conducted a study on *E. coli* transfer from foods by houseflies. About 40-60 houseflies were exposed to food contaminated by *E. coli* (8 log10 CFU/ml) for 30 min in a sterile cage. They concluded that the geometric mean carriage (log10 CFU/fly) of *E. coli* ranged from 2.25 to 3.77 CFU/fly. In the second part of the study, an E. coli-contaminated food (sugar–milk solution, an uncooked steak, and potato salad) was placed in a jar with individual houseflies for different periods to observe the amount of *E. coli* transfer to the jar's inner surfaces per landing. *E. coli* transfer for the sugar–milk was 3.5 ± 0.7 log10 CFU/fly-landing, 3.9 ± 0.7 for steak, and 2.61 ± 1.16 for the potato salad (De Jesús et al., 2004).

Transmission of human pathogens from flies to plants/vegetables/leafy greens

(Pace et al., 2017) reported that blow flies are more active than house flies in depositing *E. coli* O157:H7 and *S. enterica* on lettuce. Blow flies and house flies were introduced to lettuce plants after contact with manure contaminated with *E. coli* O157:H7- or *S. enterica*. On one hand, no significant difference was observed between the deposition of *E. coli* on lettuce by the two fly

species, even though blowflies collected significantly more *E. coli* O157:H7 than house flies from the manure. On the other hand, blow flies transferred more *Salmonella* onto the lettuce than house flies despite no significant difference in the amount of *Salmonella* picked up by both types of flies (Pace et al., 2017). Previous research found that bacteria species could attach differently to flies and plants. For instance, it has been observed that *Salmonella spp.* can attach to tomatoes and alfalfa sprouts easier than *E. coli* O157:H7 (Barak et al., 2002; Zhang et al., 2014), while *E. coli* O157:H7 can attach to banana leaves more easily than *S. enterica* (Chua & Dykes, 2013). However, Talley et al. (2009) found that house flies transferred green fluorescent protein (GFP)-tagged *E. coli* O157:H7 to spinach plants under laboratory conditions after acquiring it from manure or agar medium. Another study demonstrated that the presence of fruit (cantaloupe), enhanced *Salmonella typhimurium* survival in the fly, facilitated bacterial excretion of viable bacteria from flies, and facilitated bacterial transfer between flies. They showed that in a practical sense, house flies increase the risk of foodborne pathogen contamination in the presence of sources of bacteria (e.g., garbage bins) and accessible food (e.g., buffets) (Thomson et al., 2020). Thus, the objectives of this dissertation were to investigate the follow:

1. To determine the short-time exposure effects on bacterial transfer by fruit flies from and to food in the controlled laboratory experiments.
2. To recover and enumerate *Salmonella* and *Campylobacter spp.* from flies collected from commercial poultry farms and to determine the presence of contaminated flies collected within a 100 m range of the poultry farms with these bacteria in the field experiments.

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CHAPTER TWO

TRANSFER OF ESCHERICHIA COLI TO FOODS BY FRUIT FLIES DURING SHORT TIME EXPOSURE

Abstract

Two separate experiments were conducted to determine the transfer of *E. coli* to apple slices and bologna by fruit flies during short term exposure. Short time exposure (1, 5 and 15 min) of flies to inoculated apple slices were tested in the first experiment to determine the transfer of *E. coli* to flies from contaminated food. Level of transfer from food to flies was not affected by exposure time ($P>0.05$). In the second experiment the transfer of *E. coli* from inoculated apple or bologna slices (5 min exposure) to un-inoculated slices (1, 5 and 15 min exposure) via flies were tested. More bacteria were transferred to bologna at 1 and 5 min compared to apple slices, while the number of cells transferred did not differ at 15 min exposure. The percentage of *E. coli* transferred from inoculated food to flies was low (<0.5%) while the percentage transferred from flies to un-inoculated food was relatively high (>50%). This study determined that flies can pick up and transfer bacteria to food in short exposure times.

Introduction

Flies Carry Infectious Diseases

Pathogenic viruses (Calibeo-Hayes et al., 2003; Chakrabarti et al., 2008; Otake et al., 2004; Schurrer et al., 2005; Watson et al., 2007) bacteria (Ahmad et al., 2011; Doud et al, 2014; Fleming et al., 2014; Goush et al., 2014; Soheyliniya and Barin, 2014; Wei et al., 2014) and parasites (Balla et al., 2014; Graczyk et al., 2005; Szostakowska et al., 2004) have been found on flies. Antibiotic-

resistant bacteria have also been isolated from flies (Ahmad et al., 2011; Wei et al., 2014). Ghosh et al. (2014) established that *Enterococci* persisted throughout the life cycle of house flies to colonize the gut of the adult fly while Fleming et al. (2014) found that *E. coli* survived 12 hours in the crop and rectum of house flies. Furthermore, Wei et al. (2014) reported that *Proteus mirabilis* lasted for 3 days in the housefly digestive tract and Gill et al. (2016) established that *Campylobacter spp.* survived 1, 2, 4, 8, 12 and 24 hours after inoculation.

Flies Transmit Pathogens

Several types of flies live in close association with humans (synanthropic), including flesh flies, house flies, fruit flies and blowflies among others (Olsen, 1998). These families of flies (*Sarcophagidae*, *Muscidae*, and *Calliphoridae)* have become known as filth flies that breed in animal feces, garbage and other organic matter including food (Greenberg, 1973). Synanthropic flies that land near humans have been long recognized as vectors for numerous viruses, bacteria and protozoans causing human disease and are more prevalent where poor sanitation conditions exist, particularly in urban areas (Greenberg, 1971). Bacteria that have been transmitted by flies include *Salmonella spp., Shigella spp., Vibrio spp., Escherichia coli*, *Staphylococcus aureus*, *Campylobacter spp., Yersinia enterocolitica*, *Pseudomonas spp., Chlamydia spp.* and *Klebsiella spp.* (Echeverria et al., 1983; Kahn and Huq, 1978; Khin et al., 1989; Fotedar et al., 1992; Bidawid et al., 1978; Fukushima et al., 1979; Fotedar, 2001; Olsen, 2001; Forster et al., 2007). Synanthropic flies also transmit common food enteropathogens *Salmonella spp., Shigella spp., Campylobacter spp.* or enterohemorrhagic *E. coli* by direct contact with humans and via contamination of food (Bidawid et al., 1978). Over 50% of the house flies collected in a hospital were also found to be a reservoir for bacteria that were resistant to at 10 antimicrobial agents (Rahuma et al., 2005).

Flies can also transfer pathogens to food by direct contact with body parts, vomiting and through fly feces (Olsen, 1998). Levine & Levine (1991) reported that shigellosis could be transmitted by flies tofood and eating utensils with an inoculum as low as 10-100 cells. As a result, the U.S. FDA classifies flies as adulterants thus requiring that flies be prevented from establishments that handle food (De Jesus et al, 2004).

Flies Transmit Pathogens to Food

The capability of flies to transfer pathogens to food was determined many years ago. The relationship between the presence of flies in close proximity to food and the spread of typhoidal diarrheal disease was first identified in 1904 during the Boer and Spanish-American Wars (Nash, 1904). Ortrolenk and Welch (1942) conducted a controlled laboratory study to demonstrate that flies fed on *Salmonella enteriditis*-inoculated food can transfer the bacterium to other flies and food and that *S. enteriditis* survived in the fly throughout its 4-week lifespan. Peppler (1944) found that houseflies transported *S. enteriditis* 3 miles from a sewage pond to a kitchen and flies carried *Salmonella* from processing plants to markets and homes in Mexico (Greenberg et al., 1963). Greenberg (1964) reported that when house flies were exposed to dog feces containing 10^5 *S. typhimurium* cells/g for 2 hours, 5 out of 20 flies became infected with *Salmonella* containing between 43 and 635 bacteria per fly. The infected flies were exposed to a Mexican milk drink (Atole) for 100.5 hours which were then (surprisingly) consumed by volunteers. While none of the volunteers showed signs of illness, 8 of 10 milk samples contained between 5,000 and 640,000 *S. typhimurium* cells/ml. Other examples of flies contaminating food or food production facilities include Fukushima et al. (1979) detecting *Yersinia enterocolitica* from flies collected at a swine production facility and Rosef & Kapperud (1983) isolating *C. jejuni* from flies at both a poultry

and swine farm. Olsen and Hammack (2000) found *Salmonella enteritidis* at an egg laying operation which had produced eggs implicated in *S. enteriditis* outbreaks. Flies have been implicated as vectors in other bacterial epidemics and outbreaks including an enterohemorrhagic colitis epidemic in a Japanese nursey school had *E. coli* O157:H7 isolated from patients and flies in the area which were indistinguishable using molecular typing and the flies were traced to a nearby cattle farm (Moriya et al., 1999).

Several studies have reported that bacteria can be transferred from food to flies (Kobayashi et al., 1999; Sasaki etal., 2000; De Jesus et al., 2004). Food carrying *E. coli* O157:H7 that was consumed by flies proliferated and discharged the bacteria rapidly showing that flies are not simple vectors for pathogens but can increase bacterial numbers in the digestive tract (Kobayashi et al., 1999). Kobayashi et al. (1999) also reported that 6-7 logs of *E. coli* O157:H7 in the alimentary canal immediately after feeding an inoculated sugar solution. Sasaki et al. (2000) reported that *E. coli* O157:H7 proliferated in the mouth and crop of house flies, increasing in the number of bacteria present up to 4 hours which persisted at least 4 days after feeding.

Time Required for Transfer

Previous studies have examined contact times with the contaminating surface of longer the 30 minutes and how flies ingest bacteria then transfer it to food or other surfaces. For example, De Jesus et al. (2004) exposed flies to inoculated sugar/milk, potato salad and steak for 30 min prior to contact with clean glass jar surfaces. Greater than 1 log of *E. coli* was detected on 43, 53, and 62% of the flies exposed to sugar/milk, steak and potato salad, respectively. De Jesus et al. (2004) further reported that contaminated flies exposed to food carrying 10^8 CFU/g transferred 10^4 CFU/g of bacteria to clean surfaces (0.001%). In contrast, the current study examined contact

times with a contaminated surface of less than 15 minutes. Specifically, transfer of *E. coli* from inoculated apple to flies and from inoculated apple and bologna to un-inoculated apple and bologna during 1, 5 and 15 minutes exposure times were evaluated.

Methods

Preparation and Maintenance of Fruit Flies

Wingless fruit flies, *Drosophila melanogaster* Meigen, were purchased from Carolina Biological Supply Company, Burlington, NC as a fruit fly culture kit. Adult fruit flies were delivered and maintained at the Clemson Urban Research Laboratory, Clemson, SC. Adult flies from the culture were placed into 28.5 X 95 mm polystyrene vials (VWR Fly Vials Wide PS CS500, VWR International, Radnor, PA). Vials consisted of a feeding medium, Formula 4-24® Instant Drosophila Medium (Plain), obtained from Carolina Biological Supply Company, mixed with 10 ml of distilled water and a few granules of Fleischmann's active yeast (Associated British Foods, London, UK). Adult flies were then placed into the clean 28.5X95 mm polystyrene vials (VWR Fly Vials Wide PS CS500, VWR International) to lay eggs, and a bonded dense-weave cellulose acetate vial plug(VWR International) was used to seal the vial. Flies were reared in a 22 ^{0}C in rearing room with the fluctuation of 20.5 ^{0}C - 23.3 ^{0}C on 12-h day/night light cycle.

In order to maintain and grow fly cultures, every two weeks the same procedure was followed. Fly colonies were transferred into new polystyrene vials, which were composed of a feeding medium, 10 ml of distilled water and a few granules of Fleischmann's active yeast. Adult flies were then placed into the clean vials to lay eggs, and sealed with the vial plug and held in a rearing room at 22 ^{0}C with the fluctuation of 20.5 ^{0}C - 23.3 ^{0}C on 12-h day/night light cycle. This left the remaining eggs, larvae and pupae for future adults in the now adult fly free vial. Between 2 and 7 day old

fruit flies were separated from feeding vials and put in the vials (each with 20 fruit flies) at the Urban Entomology Research Laboratory in Clemson University just before use in eachexperiment.

E. coli Culture Preparation

A 2% ampicillin stock solution was prepared with distilled water and filter sterilized aseptically using a 0.45μm syringe filter (Acrodisc Sringe filters 0.45 μm Supor). The stock solution was kept in the refrigerator at 4 ^{0}C until needed. Sixteen g Tryptic Soy Agar (TSA) added to 400 ml distilled water in 500 ml bottles and dissolved by stirring. The media was autoclaved at 121 ^{0} C for 15 min in a steam sterilizer (Steris-Amsco Lab 250, LV 250,UK). Autoclaved TSA media was cooled to 48-50 ^{0} C in a water bath Precision, Microprocessor Controlled 280 Series Water Bath). Then, 2 ml ampicillin stock solution was mixed gently to 400 ml media before pouring aseptically in the sterile Petri dishes yielding a 0.01% ampicillin concentration in the media.

An *Escherichia coli* ampicillin-resistant strain with a fluorescent gene was used for the bacterial transfer and survival studies. A non-pathogenic *E. coli* strain JM109 was labelled with jellyfish green fluorescent protein according to the protocol as described previously (Jiang *et al*., 2002). The competent bacterial cells were electroporated in a Gene Pulser II (Bio-Rad, Hercules, CA) with plasmid vector pGFPuv (ClonTech, Palo Alto, CA). Transformants were selected from isolated colonies grown on Luria-Bertani agar (LB) plates containing100 g ampicillin/mL. The resulting ampicillin-resistant transformants emitted bright green fluorescence underUV light. The stability of GFP label in the *E. coli* strain was determined by streaking on trypticase soy agar (TSA) plates containing 100 g ampicillin/mL for several generations. The *E. coli* JM 109 culture was held in a −80˚C freezer in vials containing tryptic soy broth (Becto™ Tryptic Soy Broth, Becton Dickinson and company Sparks, MD) supplemented with 20% (v/v) glycerol (Sigma,

St. Louis, MO). The frozen vial was thawed atroom temperature prior to culturing. From this thawed vial, 0.1 mL of culture was transferred to 10 mL TSB (DIFCO) containing 0.5% ampicillin (Sigma-Aldrich, St. Louis, MO, USA) in 2 loosely screw-capped tubes and then the tubes were incubated for 16 - 18 h at 37˚C with vigorous shaking (Thermolyne Maxi-Mix III type 65,800, Barnstead/Thermolyne, Dubuque, IA). The second transfer was prepared from this first transfer culture by adding 0.1 mL from the first transfer tube to another fresh 10 mL TSB (DIFCO) with 0.5% ampicillin (Sigma), and again incubated for 16 - 18 h at 37˚C with shaking.

Actively growing cultures were used for each experiment. Growth culture was centrifuged at 5000 rpm for 15 min (Eppendorf Centrifuge 5804 R, 15 amp version, Hamburg, Germany), then the pellet re-suspended in 10 mL of sterile 20% sucrose solution to obtain a population of approximately 6 - 7 log CFU/mL. Initial cell populations were verified by enumeration of the cells following surface plating in Tryptic Soy Agar (TSA) containing 0.5% ampicillin (DIFCO™, Becton Dickinson and company Sparks, MD) and incubating at 37˚C for 24 h. A new inoculum was prepared on each day of the experiment.

Experiment 1: Contact time Effects on Transfer of E. coli from Bologna and Sliced Apples to Flies

Sliced skinless apples were placed in the Petri dishes and surface moisture of apples was patted dry with a sterile gauze. Each surface was inoculated with 0.2 ml of a 20% sucrose solution containing approximately 6 logs/ml of fluorescently-labelled and ampicillin resistant (to 0.5% ampicillin) *E. coli.* This inoculum was left to dry/attach for 5 min after which one vial of flies (20 fruit flies) were exposed to apple slice surface for 1, 5, and 15 minutes in closed Petri dishes. The flies were immediately immobilized by placement into -30 °C for 4 min. All of the flies were

transferred into test tubes containing 5 ml of 0.1% peptone water using sterile forceps then tubes were vortexed 3 times for 15 seconds. One hundred microliters of the solution and 10-fold serial dilutions were spread-plated on tryptic soy agar (TSA) plates supplemented with ampicillin. After the flies were removed, the apple slices and bologna were placed into sterile bags including 20 ml 0.1% peptone solution. After shaking for 30 second, 0.1 ml of the solution and 10-fold serial dilutions were plated on TSA supplemented with ampicillin. All plates were incubated at 37 ° C for 24 h and the number of ampicillin-resistant *E. coli* was counted with a bacterial colony counter (model 3325; Leica Quebec Darkfield). Plates were examined under UV light and only fluorescent colonies were counted. Bacterial counts were converted into colony forming units per fly (cfu/fly), log cfu/fly and % transfer of cfu from apple slice to flies. The % transfer was calculated using the following formula:

$$\%\ \text{transfer} = \frac{E.coli\ \text{recovered from flies}}{(E.coli\ \text{recovered from inoculated food slices} + E.coli\ \text{recovered from flies})}\ X\ 100$$

Experiment 2: Transfer of E. coli by Fruit Flies from Contaminated Food to "clean" Food

E. coli Transfer from Inoculated Apples to Clean Apples

Gala apples were obtained from a local store and held under refrigerated conditions (4 ^{0}C) until use within 4hours. On the dates of each experiment, the apples were removed from refrigerator to equilibrate to 22±4° C approximately 1 hour, sliced with a sterile apple slicer then placed in a sterile petri dish (60 mm diameter x 15mm deep).

The contamination of fruit flies with *E. coli* was conducted in Petri dishes. The sliced apples were placed in the Petri dishes and surface juice of the apples slices was removed with a sterile gauze. Each apple slice was inoculated with 0.2 ml of a 20% sucrose solution containing approximately 6

logs/ml of fluorescently-labelled and ampicillin resistant (to 0.5% ampicillin) *E. coli* after which the slice was held for 5 min. One vial containing 20 fruit flies were exposed to the inoculated apple slices for 5 minutes in the closed petri dishes. The contaminated flies were then transferred onto clean apple slices within the Petri dishes (no surface juice removed) then flies were allowed to walk on the apple surface for 5 min. Five minutes was chosen as the contact time since this time yielded the greatest transfer for the three times tested in Experiment 1. The flies were quickly immobilized by refrigerating the Petri dishes for 4 min and then flies were transferred into 5 ml 0.1% peptone using sterile forceps. After vortexing three times for 15 second, appropriate dilutions were made and plated TSA plates supplemented with 0.5% ampicillin. The apple slices on which the flies removed were put into the sterile bags with 20 ml 0.1% peptone solution. After shaking for 30 second, appropriate dilutions were prepared and plated TSA plates supplemented with 0.5% ampicillin. Plates were incubated at 37 ^{0}C for 24 h and the colonies counted. *E. coli* colonies were confirmed by examining plates under UV light.

E. coli Transfer from Inoculated Bologna to Clean Bologna

Turkey bologna meat obtained from a local market was held under refrigeration at 4 ^{0} C until use within 4 hours. Bologna slices were cut to the size of a Petri dish bottom (60 mm diameter) and placed in the Petri dishes then surface juice of the bologna slices was removed with a sterile gauge. Each bologna slice was inoculated with 0.2 mL *E. coli* inoculum containing 0.1 % peptone (peptone used this time instead of 20% of sugar solution) (Yee, 2003) then allowed to stand for 5 min. Twenty fruit flies were exposed to the inoculated bologna slices for 5 min in the closed Petri dishes. The contaminated flies were then transferred onto clean bologna slices within the Petri dishes (no surface juice removed for this bologna) and allowed to walk on the bologna

surface for 5 min. The flies were quickly immobilized by refrigerating the Petri dishes for 4 min and transferred into 5 mL of sterile 0.1% peptone. After three times vortexing for 15 sec, appropriate dilutions were made and plated onto TSA plates supplemented with 0.5% ampicillin. The bologna slices from which the flies removed were put into sterile bags with 20 mL of sterile 0.1% peptone solution. After shaking for 30 sec, appropriate dilutions were made and plated on TSA supplemented with 0.5% ampicillin. Plates were incubated at 37^0C for 24 h and the colonies counted. *E. coli* colonies were confirmed by examining plates under UV light. Bacterial counts were converted into colony forming units per fly (cfu/fly), log cfu/fly and % transfer of cfu from apple slice to flies.

The % transfer from inoculated food to flies was calculated using the following formula:

$$\%\ \text{transfer} = \frac{E.coli \text{ recovered from flies}}{(E.coli \text{ recovered from inoculated food slices} + E.coli \text{ recovered from flies})}\ X\ 100$$

The % transfer from flies to clean food slices was calculated using the following formula:

$$\%\ \text{transfer} = \frac{E.coli \text{ recovered from uninoculated food}}{(E.coli \text{ recovered from flies} + E.coli \text{ recovered from uninoculated food})}\ X\ 100$$

The % transfer from inoculated food to clean food slices was calculated using the following formula:

$$\%\ \text{transfer} = \frac{E.coli \text{ recovered from uninoculated food}}{(E.coli \text{ recovered from inoculated food slices} + E.coli \text{ recovered from uninoculated food})}\ X\ 100$$

Statistical Analyses

The experiment was replicated 3 times with 4 observations per treatment per replication. The treatments were exposure time (1, 5 and 15 min) and food type (apple slice and bologna). The data was analyzed to determine the effect of exposure time and food type on transfer of *E. coli* to food

by flies. The main effects of exposure time and food type and their interaction were analyzed by ANOVA using the GLM procedure of SAS (2017) to determine if the main effects had a significant effect ($P \leq 0.05$) on bacterial transfer. Descriptive statistics (mean, median, standard deviation) were calculated for $\log_{10}$ cfu/slice, percent food to fly transfer, percent fly to food transfer and percent food to food transfer. Least square difference (LSD) multiple comparison procedure was also used to evaluate significant differences ($P < 0.05$) among means.

Results

Experiment 1: Contact time Effects on Transfer of E. coli from Sliced Apples to Fruit Flies

The contact time of flies with contaminated apple slices did not significantly affect the number of *E. coli* cells recovered from flies (Table 2.1). The population of bacteria recovered from inoculated apples (used in % transfer calculation) was in the 6 log cfu/ml range which was in the same magnitude as the inoculum population used to inoculate apple slices. The % of bacteria on apples transferred to flies was below 1% which is not surprising since the surface area on flies is very small. However, there were nearly 5 logs of *E. coli* recovered from flies after walking on inoculated apples for only 1 min.

Table 2.1 Transfer of *Escherichia coli* from inoculated apples to wingless fruit flies after various exposure times

Exposure (min)	Log cfu/fly			% transmission[1]		
	mean	[2]SD	max	mean	SD	max
1 min	4.8	0.3	5.0	0.4	0.1	0.6
5 min	4.8	0.3	5.0	0.5	0.2	0.9
15 min	4.7	0.5	5.1	0.3	0.1	0.5

cfu = colony forming units. n=24

[1]% transfer calculated by $\frac{E.coli \text{ recovered from flies}}{(E.coli \text{ recovered from inoculated food slices} + E.coli \text{ recovered from flies})} X\ 100$

[2]SD =standard deviation

Experiment 2: Transfer of E. coli by Fruit Flies from Contaminated Food to "clean" Food

Based on Experiment 1, flies were allowed to contact inoculated apple or bologna slices for 5 minutes, then they were placed in contact with "clean" apple or bologna slices for 1, 5 or 15 min. An average of 4.5 to 5.2 log cfu/fly of *E. coli* on flies was recovered prior to their contact with un-inoculated food. The overall transfer from the inoculated food slices to the un-inoculated food slices was divided into two steps; 1-inoculated food to fly and 2-contaminated fly to un-inoculated food. When all contact times were pooled, bologna had greater ($p \leq 0.05$) transfer of *E. coli* to flies than apple slices (Table 2.2). The percent transfer from flies to food, and from food to food via flies were greater for bologna; but transfer from food to fly was greater for apple.

Table 2.2 Transfer of E. coli from inoculated apple or bologna slices by flies to non-inoculated slices*

	Log cfu/slice			Food to Fly Transfer (%)[1]			Fly to Food Transfer (%)[2]			Food to Food Transfer (%)[3]		
	mean	SD[4]	max	mean	SD	max	mean	SD	max	mean	SD	max
Apple	4.4[b]	0.06	5.30	0.14[a]	0.02	0.50	56.4[b]	2.60	95.30	0.11[b]	0.03	0.87
Bologna	5.2[a]	0.06	5.90	0.11[b]	0.02	0.36	68.5[a]	2.50	85.30	0.31[a]	0.03	1.01

*Flies were exposed to inoculated apple of bologna slices for 5 minutes. Values for transfer were pooled for flies contacting un-inoculated slices for 1, 5 and 15 min.

[a,b]means with different superscripts are significantly different ($p \leq 0.05$).

[1]% food to fly transfer = $\frac{E.coli \text{ recovered from flies}}{(E.coli \text{ recovered from inoculated food slices} + E.coli \text{ recovered from flies})} X\ 100$

[2]% fly to food transfer = $\frac{E.coli \text{ recovered from uninoculated food}}{(E.coli \text{ recovered from flies} + E.coli \text{ recovered from uninoculated food})} X\ 100$

[3]% fly to food transfer = $\frac{E.coli \text{ recovered from uninoculated food}}{(E.coli \text{ recovered from inoculated food slices} + E.coli \text{ recovered from uninoculated food})} X\ 100$

[4]SD =standard deviation

Apples and bologna were inoculated with approximately 6 logs of *E. coli* cfu. The transfer of *E. coli* from inoculated bologna to non-inoculated bologna was greater at 1 and 5 min exposure to flies compared to the transfer from inoculated to non-inoculated apples (Figure 2.1), possibly due to differences in surface properties. Apples had a maximum transfer of 5.3 log cycles (93.3% from flies to apples) of *E. coli* while the maximum population recovered from bologna was 5.9 logs (85.3% transfer) after 5 min exposure.

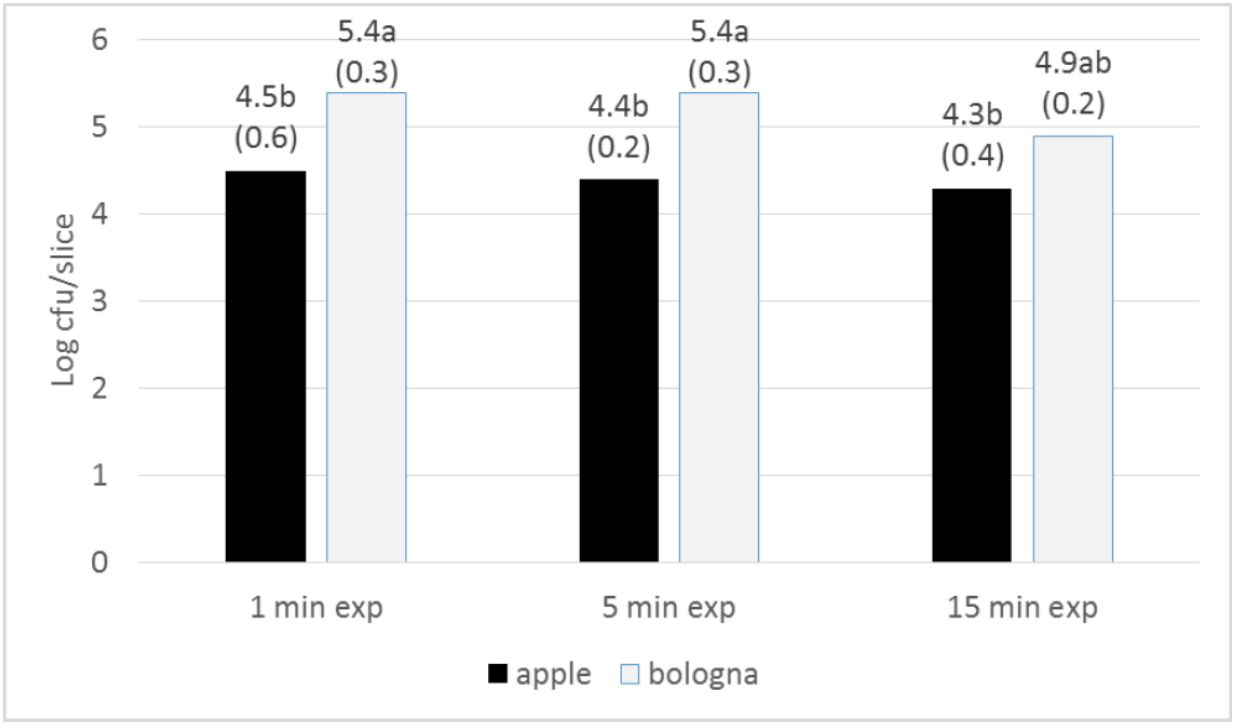

Figure 2.1 Log cfu/slice of *E. coli* recovered from apple and bologna slices after being exposed to 20 fruit flies for 1, 5 or 15 min that had been exposed to inoculated apple or bologna for 5 min.

[a,b]means with different superscripts are significantly different (P≤0.05) n=6. Standard deviations are shown in parentheses below the means.

The transfer to bologna was greater primarily due to the high percentage transferred from flies to bologna (Figure 2.2) and not due to the transfer of *E. coli* from inoculated food to flies (Table 2.2). The small surface area of fly may limit the amount transfer from the food to the fly, conversely the relatively large surface area of the uncontaminated food compared to the small contaminated surface area of the fly might allow for the high percentage of transfer from contaminated fly to uncontaminated food. Flies in contact with the food for 1 min and 5 min had greater % transfer from flies to food for bologna than flies in contact for 15 minbut % transfer did not differ for

apples due to exposure time (Figure 2.2). This indicates that flies can transfer a high percentage of bacteria from contaminated food to other foods and surfaces in a short time.

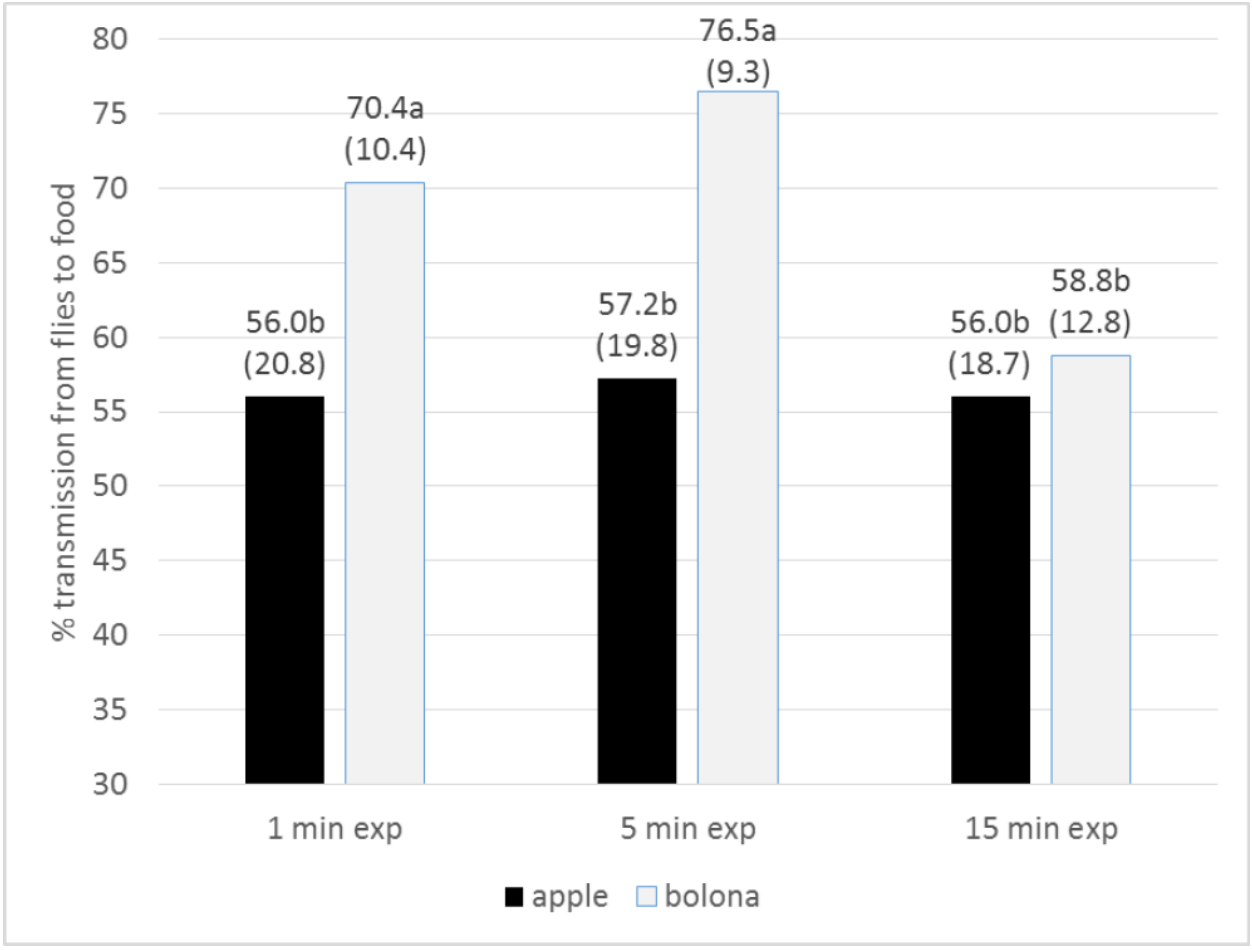

Figure 2.2 Percentage of *E. coli* transferred by flies to apple and bologna slices after being exposed to 20 fruit flies for 1, 5 or 15 min after being exposed to inoculated apple or bologna for 5 min

[a,b]means with different superscripts are significantly different (P≤0.05) n=6. Standard deviations are shown in parentheses below the means.

$$\% \text{ Transfer of E. coli from flies to food} = \frac{E.coli \text{ recovered from uninoculated food}}{(E.coli \text{ recovered from flies} + E.coli \text{ recovered from uninoculated food})} X\ 100$$

Discussion

Since most published research has examined the transfer of bacteria by flies during contact times of 30 min or more, the current study evaluated shorter contact times (1, 5, and 15 min). Previous published research that evaluated short fly contact times of less than 15 min did not test food surfaces but focused on the mechanical action of the fly during cleaning behavior. Longer contact times allow for the fly digestive system to be more involved in transfer of bacteria. Olsen (1998) determined that, in addition to contact of body parts, flies transfer bacteria via the digestive tract through vomitus and feces. Sasaki et al. (2000) reported that excreted droplets from house flies increased from 4 logs/droplet 1 hour after feeding to 5 logs/droplet at 3 hours after feeding. Thus, shorter contact times would be more impacted by contact of body parts on transfer bacteria from contaminated surfaces to flies and from flies to food. In one of the few short-term studies done to date, Jacques et al. (2017) found the cleaning behavior of house flies and fruit flies resulted in the mechanical transmission of *E. coli* to sterile Petri plates after 5 min exposure to inoculated plates. Gill et al. (2016) reported that house flies transmitted *Campylobacter jejuni* primarily by contact rather than through the digestive system even after ingestion. Contamination of food surfaces by flies requires that flies first pick up microorganisms from a contaminated surface then transfer this contamination to another food surface. To have greater control of fly contact with contaminated and un-contaminated food, wingless flies were used for the current study. Thus, we first examined the effect of short term (1, 5 and 15min) contact of flies with a contaminated surface and found that there was no difference in the number of bacteria picked up by flies between these contact times. The percentage of bacteria transferred to flies was very low (≤ 0.5%) however with a highly contaminated surface as was used in this study, there were nearly 5 log cycles of bacteria transferred to flies from the contaminated surface. De Jesus et al. (2004)

recovered from 2.3 to 3.8 log population of bacteria from flies after exposure to sugar-milk, potato salad or uncooked steak for 30 min previously inoculated with 8 logs of *E. coli* O157:H7. The estimated percentage of bacteria transferred in the De Jesus et al. (2004) study (~0.01%) was even lower than in the current study (0.1 to 0.5%) and illustrates the relatively high number of bacteria that can be transferred when the source surface is highly contaminated even with very low transfer rates. Flies are seeking nutrients which are often highly contaminated locations where food, animal and human waste are found. This was verified by Forster et al. (2007)who found that flies collected from a dog pound, poultry house, cattle barn, horse stable and pigpen were carriers of multiple species of microorganisms, including pathogenic *E. coli* strains. Food pathogens do not cause illness in flies allowing flies to be carriers and vectors of food pathogens. In the second experiment, the current study measured the transfer of *E. coli* from inoculated food to un-inoculated food by wingless fruit flies. The % transfer of bacteria from the inoculated food to flies was again relatively low (<0.2%) yet comparatively, the transfer from the flies to the un-inoculated food was over 50 times higher (>50%). Janisiewicz et al (1999) tested the transfer of *E. coli* to flies by exposing fruit flies to inoculated apple juice in filter paper for 2, 6, 24 and 48 hours and the transfer from flies to apple wounds by exposing the contaminated flies to apples for 7, 24 and 48 hours. Flies were contaminated with 8 log cfu/fly of *E. coli* after 2 hours and with 10 log cfu/fly by 6 hours of contact with inoculated filter paper. The inoculated flies then transferred nearly 4 to 6 log cfu/apple wound after 24 and 48 hours of exposure to food. Similar transfer of *E. coli* from contaminated apple to un-contaminated apple were found with relatively short exposure times of 1 and 5 min in the current study as were reported by Janiseiwicz et al. (1999).

Thus, short time exposure of flies to contaminated food and subsequent short time exposure to uncontaminated food results in transfer of relatively high numbers of bacteria Baloney has more transfer rate likely due to the surface properties.

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CHAPTER THREE

TRANSFER OF BACTERIA PATHOGENS BY FLYING INSECTS COLLECTED FROM POULTRY FARMS

Abstract

Filth flies, especially house flies, can harbor and ultimately distribute human pathogens to food and food contact surfaces. To determine the potential of flying insects collected from poultry grow out houses to carry *Salmonella* and *Campylobacter*, a total of 2164 flies were caught on poultry farms located in the Upstate, Middle, and Coastal regions of South Carolina and segregated based on fly family type. Capture flying insects included house flies in the family Muscidae inside the poultry house [in-HF] (N = 289), house flies just outside the poultry house [out-HF] (N = 1023), and house flies 100 meters from the poultry houses [100m-HF] (N = 547). Other flying insects included wasps in the family Vespidae species (spp.) captured just outside the poultry house [out-Vespidae] (N = 71), Vespids spp. 100 meters from the poultry house [100m-Vespids] (N = 126), flesh flies in the family Sarcophagidae just outside the poultry house [out-Sarcophagids] (N = 13), and flesh flies 100 meters from the poultry house [100m-Sarcophagids] (N = 9), blow flies in the family Calliphoridae 100 meters from the poultry house (100m-Calliphorids), darkling beetles in the family Tenebrionidae just outside the poultry house [out-DB] (N = 30), and darkling beetles 100 meters from the poultry house [100m-DB] (N = 56). Populations of *Campylobacter spp., Salmonella spp.,* and total aerobic microorganisms (APC) were recovered from the flies as well as the number of *Salmonella spp.* and *Campylobacter spp*. positive flies at a 100 m distance from the farms. Along with fly groups, chicken feces in the houses [CF] from three farms, cow manure around farm 1 and farm 2, and dog feces around farm 1 were also sampled. While no

Campylobacter jejuni was detected from any of the samples, including fly groups, chicken feces, cow manures, and dog feces, *Campylobacter coli* positive samples were detected in the cow manure samples in both replications, 100m-Calliphoridae, out-HF and 100m-DB in one out two replications on farm 2. Moreover, positive Serogroup B *Salmonella spp.* were determined in the groups in-CF, in-HF, and out-HF on farm 2 and positive Serogroup C *Salmonella spp.* were determined in the groups of in-CF, out-HF, and 100m-HF on farm 3. Data demonstrates that house flies may be a vector in the transmission of *Salmonella spp.* from poultry farms.

Introduction

The United States is the top producer of chicken meat in the world (Shahbandeh, 2022). *Salmonella* and *Campylobacter* are commonly found in the gastrointestinal tract of chickens, and poultry meat has been cited as the leading source of human salmonellosis and campylobacteriosis in the United States (Thames & Sukumaran, 2020). According to the FoodNet 2021 report, the number of infections decreased by 8%, and incidence was unchanged for *Campylobacter*, while incidence decreased for *Salmonella* compared with the 2016-2018 average. However, the report highlighted that substantial efforts are still needed to improve food safety and to achieve national goals, especially for *Salmonella* and *Campylobacter*. The report declared 22,019 infections, 5,359 hospitalizations, and 153 deaths by pathogens in the U.S. in 2021. *Campylobacter* and *Salmonella* caused to highest infection rates, with the number of 8974 and 7148, respectively; in other words, *Campylobacter* caused 17.8 cases per 100,000 population while *Salmonella* caused 14.2 cases per 100,000 population (Collins et al., 2022). *Campylobacter* and *Salmonella* are the two primary pathogens causing human gastroenteritis related to poultry meat consumption (Rouger et al., 2017). Globally, it has been estimated that

there are estimated 94 million non-typhoidal *Salmonella* (NTS) cases each year of which 80.3 million cases are linked to foodborne origins that result in 155,000 deaths each year (Majowicz et al., 2010; Mouttotou et al., 2017). Animal sources cause about 46.4% of human *Salmonella* infections, and poultry has been implicated in a majority of these infections (Sanchez et al., 2002). Based on the FoodNet 2021 report, the five most common *Salmonella* serotypes causing foodborne illnesses are *enteritidis*, *newport, typhimurium, javiana*, and *i 4,[5],12:i:-* since 2010 (Collins et al., 2022). *Salmonella* infections in poultry can be from both vertical and horizontal transmission (Dar et al., 2017). Environmental sources, such as rodents, wild birds, feed, insects, transportation, and farm environment, may cause a *Salmonella* outbreak by horizonal transmission in a poultry flock, while transmission from hen to offspring (vertical transmission) is less common (Bailey et al., 2001).

As stated previously, contaminated poultry products are also the main source of human *Campylobacter spp*. infections and create more than 50% of all cases (EFSA, 2014; Gill et al., 2017; WHO, 2013). While *C. jejuni* is the major strain implicated in more than 90% the cases, *C. coli* is the second most frequently isolated species accounting for 5-10% of cases (Gillespie et al., 2002). Horizontal transmission from environmental sources including contaminated water, feed, insects, rodents, and specifically house flies (*Musca domestica*) were shown as the main route of *Campylobacter spp.* transmission in poultry houses (Agunos et al., 2014; Gill et al., 2017; Hermans et al., 2012; Newell & Fearnley, 2003; Sahin et al., 2002; WHO, 2013).

The U.S. Department of Agriculture has identified forty-seven species of flies categorized as "filth flies" that might disseminate foodborne pathogens. Twenty-one of 47 species are a potential threat to human health and classified as "disease-causing" flies. Filth flies including flesh flies (Sarcophagidae), house flies (Muscidae), fruit flies (Drosophilidae) and blowflies (Calliphoridae)

live in close association with humans (synanthropic) and can quickly infest human populated areas with unsanitary conditions, such as animal feces, garbage, and other decaying organic matter (Olsen, 1998).

The synanthropic house fly, *Musca domestica (Diptera: Muscidae),* is a potential reservoir and transmitter for a diverse range of pathogens (bacteria, fungi, viruses, and parasites), some of which lead to diseases in humans and animals. In fact, Khamesipour et al. (2018) confirmed more than 130 pathogens were isolated from the house flies. House flies can harbor as many as 100 species of bacteria ranging between 10^7 and 10^{10} CFU per fly (Chifanzwa & Nayduch, 2018; Greenberg, 1973). Houseflies may transfer pathogens by the sponging of mouthparts and on the body surface (particularly leg hairs and on the sticky parts of the feet), regurgitation of vomitus, and the alimentary tract (Olsen, 1998; Rosef & Kapperud, 1983; West, 1951).

Numerous studies have demonstrated that house flies can carry and transfer *Salmonella* and *Campylobacter*. For example, in a controlled study, Shane et al., (1985) demonstrated that house flies transferred *C. jejuni* between chicken flocks and Wasala et al. (2013) reported that house flies carried and deposited E. coli 0157:H7 onto spinach. Ommi et al. (2017) detected *Salmonella spp.* and *Campylobacter spp.* from houseflies caught in kitchens, animal farms, hospitals, and slaughterhouses. Choo et al. (2011) reported that house flies carried *Campylobacter spp.* and *Salmonella spp. on a poultry farm and* Hald et al. (2004) found that hundreds of flies passed through the ventilation system into broiler house and *Campylobacter spp*. positive flies were captured in the house. Furthermore, Rosef & Kapperud, (1983) reported that flies carried *Campylobacter spp.* from a chicken and swine farm.

The objective of the present study was to recover and enumerate *Salmonella* and *Campylobacter* spp. from flies collected from commercial poultry farms, and to determine presence of contaminated flies collected within a 100 m range of the poultry farms.

Material and Methods

Collection of Flies

Flies were collected on two separate occasions (two replications) from August to October from each of three commercial broiler farms. The farms were in three different regions of South Carolina, US-Farm was in the Upstate (Figure 3.1); Farm 2 was located in the Midlands (Figure 3.2); and Farm 3 was located near the Coast in the Low Country (Figure 3.3). Farm 1 had three houses and 3 traps were set up outside each house on farm 1. Farm 2 had 8 poultry houses and four traps were set up outside the houses (House number 2, 4, 5, and 7). Farm 3 had 4 houses, so two traps were hung outdoor of these houses (House number 2 and 3, where are at the center of 4 houses).

All sample collection was completed while the same flocks were housed.

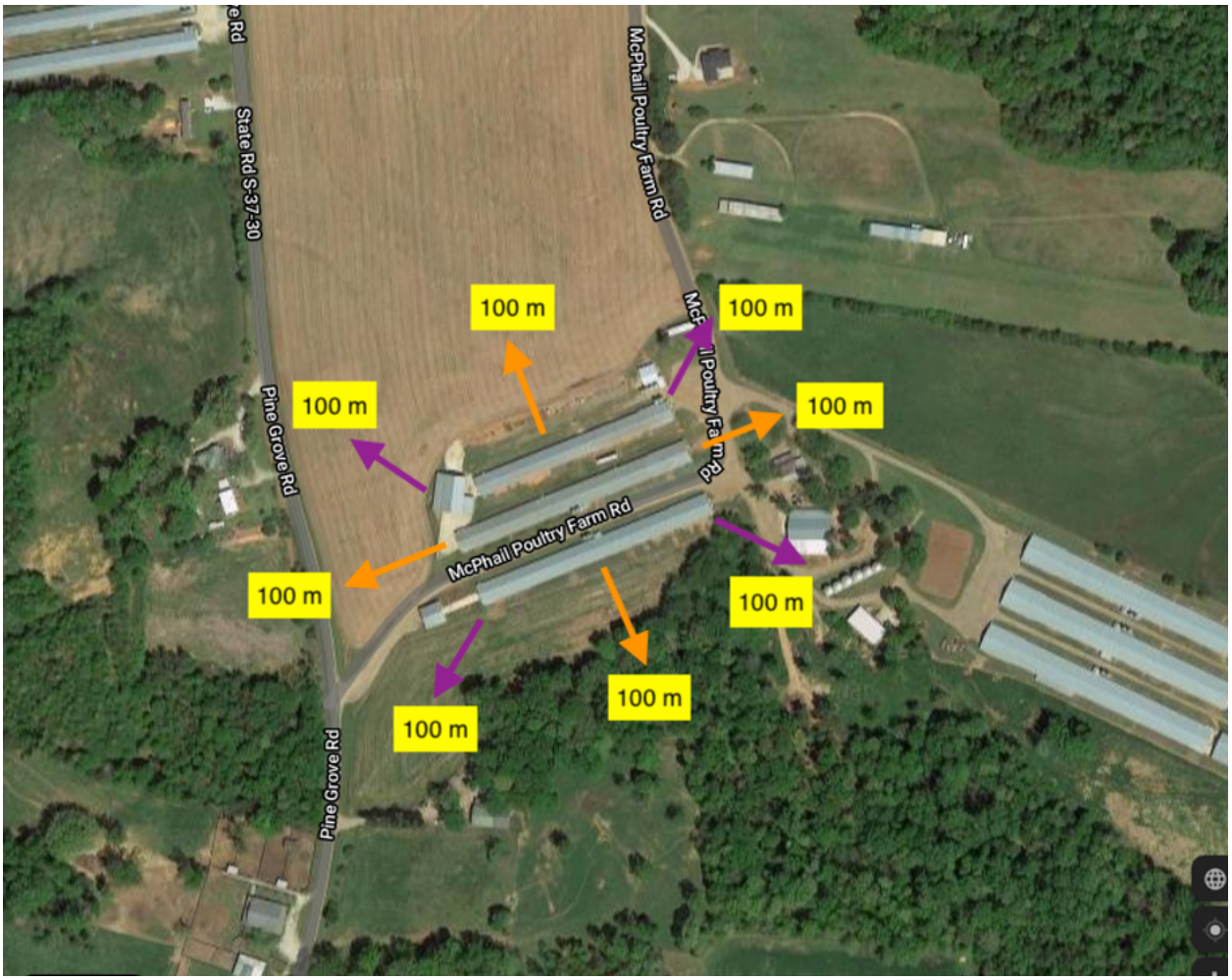

Figure 3.1 Farm 1 in Upstate, South Carolina, US

GSP coordinates: 34°33'32.3"N 82°56'17.4"W

Orange arrows show the first replication, and purple arrows indicate the second replication of the fly trap locations

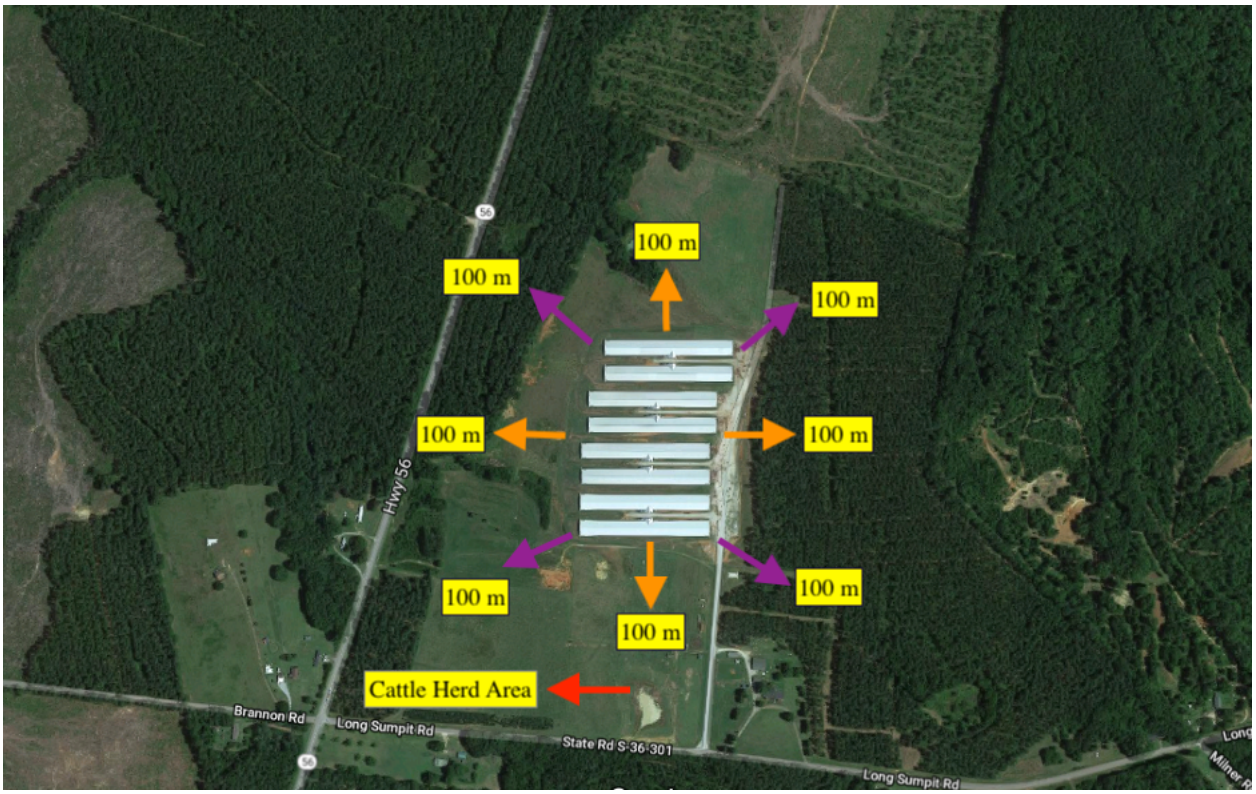

Figure 3.2 Farm 2 in Midlands, South Carolina, US

GPS coordinates: 34.21549074559753, -81.87741246783675

Orange arrows show the first replication, and purple arrows indicate the second replication of the fly trap locations

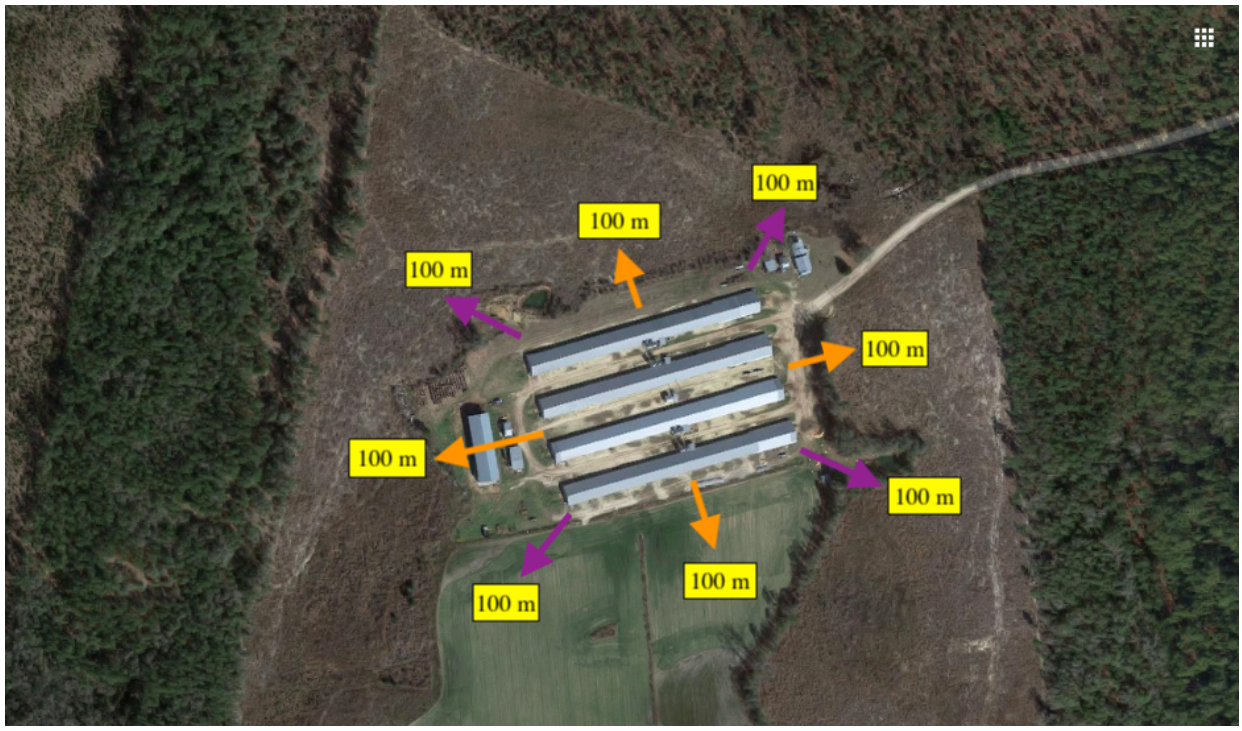

Figure 3.3 Farm 3 in Coastal, South Carolina, US

GPS coordinates: 34°18'17.3"N 79°12'28.5"W

Orange arrows show the first replication, and purple arrows indicate the second replication of the fly trap locations

Figure 3.4 shows fly trap set up and collection times (sampling). At each sampling session, on day zero, yellow sticky fly traps (Starbar EZ Trap-Wellmark International, USA) were set 1.8 m high above the ground inside & outside the grow-out houses on the farm. Vigoro shepherd hooks (from Home Depot-Atlanta, GA USA) were used to place the fly traps at locations 100-meters from the growout houses in 4 quadrants. Fly trap locations are shown in Figure 3.5-Farm 1, Figure 3.6-Farm-2 and Figure 3.7-Farm 3. Four traps were placed 100 meters from the center of the farm in each direction (north, south, east, and west). Flytraps were left for two weeks before collection. The chickens were 1-2 weeks old when the traps were first set. For the second replication another set of flytraps were placed in the northeast, northwest, southeast, and southwest quadrants also

away 100 meters from the center of the farm, as well as two traps near the house (one inside on the right-center and one outside on the right corner of the house).

Also, chicken feces samples were collected from 6 different places in the houses using sterile gauze (put the gauze on the dropping, step on it with a clean boot cover and collect it) from each house on the farm and placed sterile bags.

Additionally, cow manure samples around Farm 1 and Farm 2, dog feces around Farm 1 were collected.

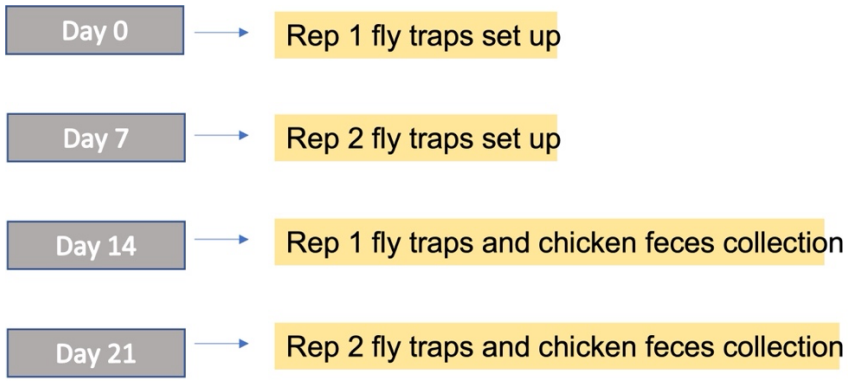

Figure 3.4 Fly trap set up and collection times (sampling)

These steps were followed for each farm except the farm 3. We could not visit this farm on the day 14 because of a hurricane and flooding in this area, so the first set of fly traps was collected one week later, and one-time chicken feces were collected on the day 21 after placing the fly traps. Fly collection was completed at the end of three weeks for each farm. Collected flies were pooled based on the fly species and locations (indoor, outdoor, and 100 m) where they were captured. For example, the group of house flies obtained from indoor are called in-HF. The number and weight of flies were recorded before analysis.

Sample Categorization:

Indoor: All the flies caught in the houses on a farm were pooled based on the species of flying insect.

Outdoor: All the flies caught outside of the houses were on a farm were pooled based on the species of flying insect.

100 m: All the flies collected 100 m away were pooled again based on species of flying insect (North, South, West, and East for the rep 1) and (Northeast, Southeast, Northwest, and Southwest for rep 2).

Chicken feces (CF): All gauze samples were pooled from different houses on each farm for each replication.

The pooled flies based on species and catch locations were transferred in sterile tubes and crushed using sterile forceps, then 0.1% peptone water added according to the weight of flies, (9 ml per gram fly) and vortexed. Pooled gauze samples from each farm were weighed and mixed with 0.1 % peptone water for the recovery (225 ml peptone water to 25-gram sample). Serial dilutions were prepared for the total count, *Salmonella* plate count, and *Campylobacter* plate count.

After serial dilutions, samples were pipetted onto 3M Petrifilm Aerobic Count Plates, XLT-4 CM1061; Oxoid) plates with XLT-4 selective supplement, and *Campylobacter* Blood-Free Selective Agar [modified charcoal-cefoperazone-deoxycholate agar (mCCDA)] plates (CM0739; Oxoid) with CCDA Selective Supplement (SR0155; Oxoid).

Aerobic count and *Salmonella* plates were incubated at 35°C for 48 hours and at 37°C for 24 hours, respectively before counting. *Campylobacter* plates were put in 5-10% CO_2 incubator at 42°C for 48 hours before enumerating.

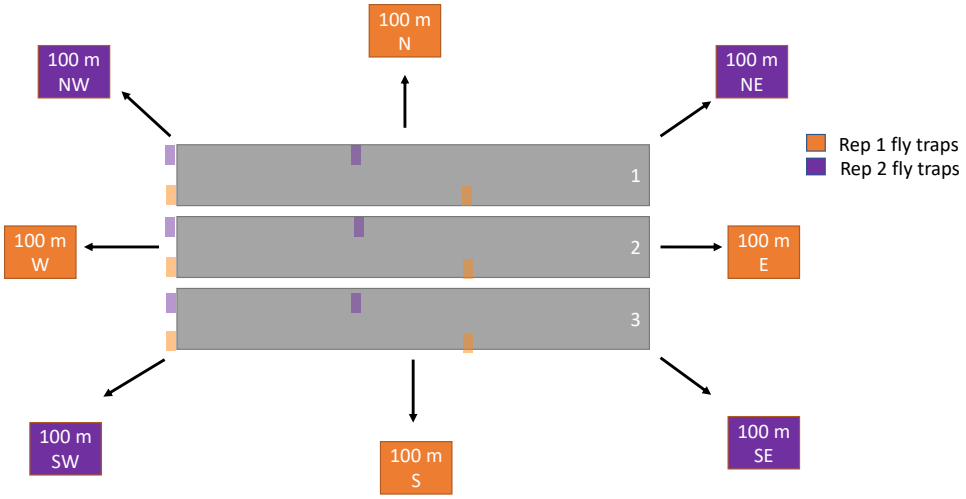

Figure 3.5 Fly trap locations around the Farm 1 in Upstate, South Carolina, US

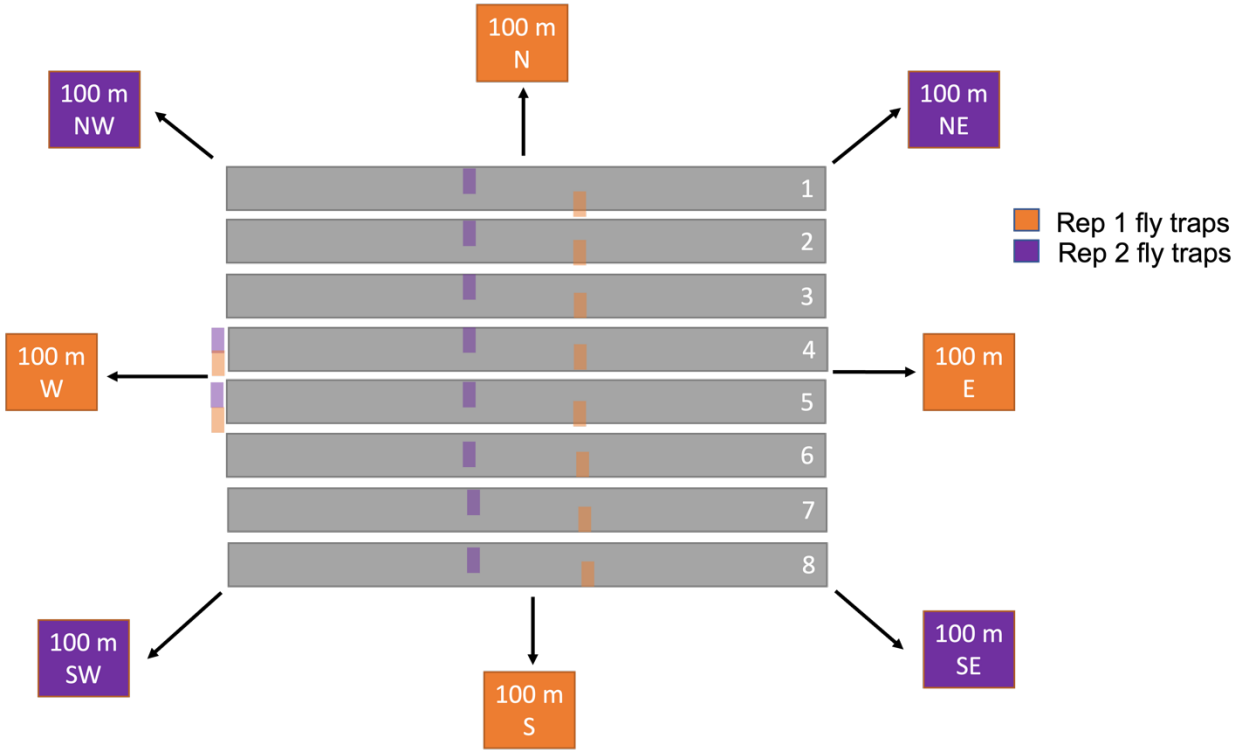

Figure 3.6 Fly trap set up locations around the Farm 2 in the Midlands, SC, US

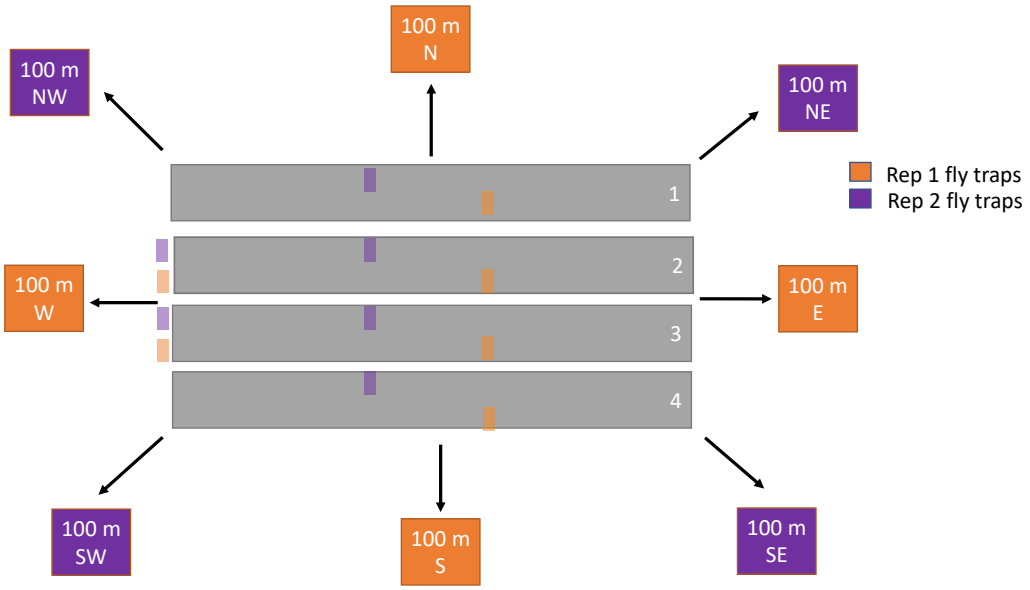

Figure 3.7 Fly trap locations around the Farm 3 in Coastal, SC, US

Salmonella and Campylobacter Isolation and Identification

For *Salmonella spp.* isolation, the samples in 0.1% peptone water were incubated at 37 °C for 24 h. Then, 0.1 mL of the pre-enriched sample was added to 10 mL of Rappaport-Vassiliadis (RV) enrichment broth (CM0669; Oxoid) and incubated at 37 °C for 24 h. A loopful of RV enrichment broth culture was streaked onto XLT-4 plates including XLT-4 selective supplement and incubated at 37 °C for 24 h. Purified colonies obtained after at least three streaks on XLT-4 plates were tested using the polymerase chain reaction (PCR). *Salmonella* positive samples were tested for agglutination using the Wellcolex color *Salmonella* kit (Wellcolex, UK).

For *Campylobacter spp*. isolation, 1 ml of sample in 9 ml Bolton Broth (CM0983; Oxoid) supplemented with horse blood and Bolton Broth Selective Supplement SR0183 was incubated for 4 hours at 37°C, followed by further incubation at 42°C for 24 h. Then, the broth was streaked

onto modified CCDA agar (mCCDA) previously prepared and incubated in 5-10% CO_2 incubator at 42°C for 48 h. Purified individual colonies were obtained by streaking at least three times onto modified CCDA.

Polymerase Chain Reaction (PCR)

The primers used for amplification of each of these organisms and their target genes are shown in Table 3.1.

Table 3.1 Primers used in PCR for detection of *Salmonella* and *Campylobacter jejuni* from the samples

Bacteria (target gene)	Primer sequence	Band size (bp)	Reference
Salmonella (invA gene)	F: 5'-GTGAAATTATCGCCACGTTCGGGCAA-3' R: 5'-TCA TCGCACCGTCAAAGGAACC-3'	284	(Malorny et al., 2003)
Campylobacter jejuni (mapA)	MDmapA1 upper primer 5'-CTA TTT TAT TTT TGA GTG CTT GTG -3' MDmapA2 lower primer 5'-GCT TTA TTT GCC ATT TGT TTT ATT A -3'	589	(Denis et al., 2001)

A loop full of pure culture was mixed with 500 µl sterile water, boiled for 7 min in the boiled water, centrifuged (10,000 rpm, 5 min) and the resultant supernatant was used as template DNA (4.5 µl) for PCR.

PCR amplifications (LightCycler 96, Roche) were performed individually for the *Salmonella* invA genes) and for the *Campylobacter jejuni* (mapA genes) in 25 µl reaction mix comprising of nuclease-free water (7 µl), 2x mastermix with standard buffer (Biolabs, New England) (12.5 µl), forward and reverse primers (Sigma) 0.5 µl each, and template DNA (4.5 µl).

Thermal cycling conditions for *Salmonella spp.* is consisted of initial denaturation (94°C for 120 s), followed by 45 cycles of denaturation (94°C for 20 s), annealing (64°C for 15 s), and extension (72°C for 30 s) followed by final extension (72°C for 300 s).

Thermal cycling conditions for *Campylobacter jejuni* consisted of initial denaturation (94°C for 120 s), followed by 45 cycles of denaturation (94°C for 30 s), annealing (59°C for 15 s), and extension (72°C for 60 s) followed by final extension (72°C for 300 s). Amplified products were separated by agarose gel (1%) electrophoresis (Biorad). Electrophoresis was conducted at 110 volts for 1 h in 1 x TBE buffer. Ethidium bromide was used for staining and image was obtained with a Spectroline Select Series UV Transilluminator, (Thermofisher).

Statistical Analyses

Data were entered and managed in Microsoft Excel™ 2022.

Fundamental descriptive analyses (Average, standard deviation) were determined for the *Salmonella* plate, *Campylobacter* plate, and Aerobic Plate Count and described as log CFU/fly for flies and log CFU/g for chicken feces. The raw data collection was completed in 21 days with two replications for three broiler farms. Fly samples were divided into groups based on the location and family types. One sample was created for chicken feces coming from the houses in each specific farm. For each sample, two observations were conducted per replication, and four readings were recorded.

Results

Outside temperature, humidity, and wind speed data were obtained online and corresponded to the dates of the study (https://www.wunderground.com/) (Table 3.2). Data covers all sample collection times per farm (21 days).

Table 3.2 Outdoor temperature, humidity, and wind speed on the farms.

Farms	**Temperature (°C)**			**Humidity (%)**			**Wind Speed (mph)**		
	Max[1]	Ave[2]	Min[3]	Max	Ave	Min	Max	Ave	Min
Upstate	36	23.5	14	100	68.7	24	17	3.7	0
Middle	36	26.3	14	96	69.5	24	25	6.1	0
Coastal	34	25.6	16	100	75.6	34	33	6.5	0

n=21

[1]Max refers to the maximum value recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]Min refers to the minimum value recorded during the study.

Temperatures in the houses on the farms were shown in table 3.3.

Table 3.3 Temperatures in the houses on the farms.

Farms	**Temperature (°C)**		
	Max[1]	Ave[2]	Min[3]
Upstate	30	26.9	23
Middle	34	30.8	28
Coastal	30	28.4	27

[1]Max refers to the maximum value recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]Min refers to the minimum value recorded during the study.

Chicken Feces (CF)

Average *Salmonella spp., Campylobacter spp.*, and total aerobic bacteria (log CFU/g) recovered from the chicken feces in the farms are shown in Table 3.4. Average *Salmonella spp.* and APC counts were similar on all three farms. *Salmonella spp.* in Upstate, Midlands, and Coastal farms was 9.4, 9.0, and 9.5, and APC counts were 10.3, 10.3, and 10.6 log CFU/g, respectively. However, chicken feces from the farm in the Midlands had almost a 0.7 log greater *Campylobacter spp.* than the farm in the Upstate. Also, the feces from the Coastal farm had almost 1 log greater *Campylobacter spp.* than the feces from the farm located in the Upstate. Average *Campylobacter spp.* numbers in the feces are 6.3, 7.1, and 7.3 log CFU/g in the CF in Upstate, Midlands, and Coastal, respectively.

Table 3.4 Average *Salmonella spp., Campylobacter spp.* and Total Aerobic Bacteria (Log CFU/g) recovered from the chicken feces in Farm 1, 2, and 3.

Farms	**Salmonella Log (CFU/g)**				**Campylobacter Log (CFU/g)**				**Aerobic Count Log (CFU/g)**			
	Rep 1[1]	**Rep 2[1]**	**Ave[2]**	**STD[3]**	**Rep 1**	**Rep 2**	**Ave**	**STD**	**Rep 1**	**Rep 2**	**Ave**	**STD**
Upstate	9.9	8.8	9.4	0.8	7.4	5.2	6.3	1.6	10.7	10.0	10.3	0.5
Middle	9.0	8.9	9.0	0.1	7.8	6.3	7.1	1.1	10.1	10.5	10.3	0.3
Coastal	[4]No	9.5	9.5	0.0	No[4]	7.3	7.3	0.0	[4]No	10.6	10.6	0.0

[1]Rep refers to the replication recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]STD refers to standard deviation.

[4]No refers to no sample collection due to hurricane.

Farm 1 (Upstate)

Average *Salmonella spp., Campylobacter spp.*, and APC counts (log CFU/fly) recovered from the fly species in Farm 1 are shown in Table 3.5. The dominate species of flying insect collected from

farm 1 was the house fly, *M. domestica*. With no flies captured inside the houses, a total of 547 flies created six groups, including flies just outside the poultry house [out-HF] (N = 235), flies 100 meters from the poultry houses [100m-housefly] (N = 258), Vespidae just outside the poultry house [out-Vesp] (11), Vespidae 100 meters from the poultry house [100m-Vesp] (21), flesh flies just outside the poultry house [out-Sarcop] (N = 13), and flesh flies 100 meters from the poultry house 100m-Sarcop were captured in two replications. House flies in the treatment group 100m-HF contained fewer cells of *Salmonella spp.* (almost two logs lower) than the flies discovered outside the house in the out-HF treatment group (3.9 and 5.9), and nearly 1 log higher *Campylobacter spp.* (5.0 and 4.0), and about half a log higher APC (7.6 and 7.2).

While no *Salmonella spp.* and *Campylobacter spp.* were detected in both vespid groups (out and 100 m), an average of 7.6 log CFU/fly in the out-Vesp and 8.3 log CFU/fly in the 100m-Vesp were detected in APC plates.

Sarcophagidae flies were only captured during the second replication.

While no *Salmonella spp.* were detected in 100m-Sarcop plates, 7.0 log CFU/fly *Salmonella spp.* were recovered from flies in the out-Sarcop treatment group. Also, out-Sarcop was less than 1 log higher than 100m-Sarcop (3.1 and 2.4) in average *Campylobacter spp.* count. However, the APC count in out -Sarcoph was lower than 100m-Sarcop (7.9 and 8.8 log CFU/fly), with almost one log difference in the average APC count.

All presumptive *Campylobacter spp.* and *Salmonella spp.* plates for this farm were determined to be *Campylobacter jejuni* and *Salmonella spp.* free in the PCR testing.

Table 3.5 Fly numbers, Average *Salmonella*, *Campylobacter* and Total Aerobic Bacteria (Log CFU/fly) recovered from the fly species in Farm 1 (Upstate).

Location/ Fly type	Fly numbers			Salmonella Log (CFU/fly)				Campylobacter Log (CFU/fly)				Aerobic Plate Count Log (CFU/fly)			
	Rep[1] 1	Rep[1] 2	total	Rep 1	Rep 2	Ave[2]	STD[3]	Rep 1	Rep 2	Ave	STD	Rep 1	Rep 2	Ave	STD
out-HF	115	120	235	4.4	7.5	5.9	2.2	4.0	3.9	4.0	0.1	6.2	8.2	7.2	1.4
100m-HF	128	130	258	4.4	3.5	3.9	0.6	4.6	5.5	5.0	0.6	7.2	8.1	7.7	0.7
out-Vesp	5	6	11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.4	7.7	7.5	0.2
100m-Vesp	6	15	21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.6	9.0	8.3	1.0
out-Sarc	0	13	13	0.0	7.0	7.0	0.0	0.0	3.1	3.1	0.0	0.0	7.9	7.9	0.0
100m-Sarc	0	9	9	0.0	0.0	0.0	0.0	0.0	2.4	2.4	0.0	0.0	8.8	8.8	0.0

Out: outdoor, HF: Housefly, Vesp: Vespidae, Sarcoph: Sarcophagidae.

[1]Rep refers to the replication recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]STD refers to standard deviation.

Farm 2 (Midlands)

Average *Salmonella spp*., *Campylobacter spp.*, and APC counts (log CFU/fly) recovered from the fly species in Farm 2 are shown in Table 3.6. Again, the house fly was the dominant type around the farm, especially outdoors. This time indoor house flies were also collected. A total of N = 1425 flies were caught and created five groups based on the locations and fly type, including in-HF (N = 289), out-HF (N = 770), 100m-HF (N = 245), out-Vesp (N = 50), and 100m-Vesp (N = 71) in two replications.

Average *Salmonella spp*. count in-HF, out-HF, and 100m-HF were between the 6-7 log range and close to each other with the numbers 6.3, 6.2, and 6.9 log CFU/fly, respectively. Average APC numbers between the HF groups also gave a similar pattern and were 7.5, 7.4, and 7.9 log CFU/fly. However, *Campylobacter spp.* recovered from flies collected inside the house was lower (3.8 log CFU/fly) than the *Campylobacter* recovered from flies collected outside the house- out-HF (log 5.5 CFU/fly) and 100m-HF (log 5.2 CFU/fly). These differences were traced back to replication

2, where in-HF had 2.55 log CFU/fly *Campylobacter spp.* while out is 5.9 CFU/fly and 100m-HF is 4.8 log CFU/fly.

The average *Salmonella spp.* log counts for out-Vesp and 100m-Vesp were the same (6.0 log CFU/fly), and similarly, little difference was found in the average APC and *Campylobacter spp.* count between these same treatments (out-Vesp and 100m-Vesp), with log 3.8 and 3.4 CFU/fly for *Campylobacter spp*., respectively and log 8.4 and 8.2 for APC, respectively.

There was a small herd of cattle near the poultry houses located approximately 400-500 m away. Thus, cow manures were also collected from this farm during both replications.

Table 3.6 Fly numbers, Average *Salmonella, Campylobacter spp* and Total Aerobic Bacteria (Log CFU/fly) recovered from the fly species in Farm 2 (Midland).

Location/	Fly numbers			Salmonella Log (CFU/fly)				Campylobacter Log (CFU/fly)				Aerobic Count Log (CFU/fly)			
Fly type	Rep[1] 1	Rep[1] 2	total	Rep 1	Rep 2	Ave[2]	STD[3]	Rep 1	Rep 2	Ave	STD	Rep 1	Rep 2	Ave	STD
in-HF	159	130	289	6.1	6.5	6.3	0.2	5.1	2.5	3.8	1.8	7.6	7.3	7.5	0.3
out-HF	503	267	770	6.2	6.1	6.2	0.1	5.0	5.9	5.5	0.6	7.3	7.3	7.3	0.0
100m-HF	160	85	245	7.1	6.8	6.9	0.2	5.6	4.8	5.2	0.6	8.0	7.8	7.9	0.2
out-Vesp	29	21	50	6.1	5.8	5.9	0.2	3.1	4.4	3.8	0.1	8.0	8.8	8.4	0.6
100m-Vesp	49	22	71	6.7	5.2	6.0	1.1	3.7	3.2	3.4	0.4	8.4	7.9	8.2	0.3

In: indoor, out: outdoor, HF: Housefly, Vesp: Vespidae.

[1]Rep refers to the replication recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]STD refers to standard deviation.

All presumptive *Campylobacter spp.* plates on the farm were found to be *Campylobacter jejuni* free. However, *Campylobacter coli* positive samples were detected in the cow manure samples collected approximately 400-500 m in distance and from 100m-Calliphoridae sample in the first

replication. *Campylobacter coli* samples also detected in the second replication, which were cow manure collected in the same location as the first replication, out-HF and 100m-DB (Table 3.7).

Table 3.7 Positive *Campylobacter spp.* samples from Farm 2 (Midland).

Sample	**Rep 1**[1]	**Rep 2**[1]
Cow manure	*C. coli*	*C. coli*
Out-HF		*C. coli*
100m-Callip	*C. coli*	
100m-DB		*C. coli*

Out: outdoor; HF: Housefly; Callip: Calliphoridea; DB: Darkling Beetle.

[1]Rep: Replication

Salmonella spp. PCR and agglutination results from three farms are shown in Table 3.8. In-CF and in-HF in both replications and out-HF in replication two were determined to be *Salmonella spp.* positive. They were found in Serogroup B *Salmonella spp.* by the agglutination test, and all appeared to be identical.

Table 3.8 *Salmonella* PCR and agglutination test results.

	Farms					
Location / Sample	**Upstate**		**Midland**		**Coastal**	
	Rep 1[1]	**Rep 2**[1]	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**
in-CF	Negative	Negative	Serogroup B	Serogroup B	Serogroup C	Serogroup C
in-HF	No HF	No HF	Serogroup B	Serogroup B	No HF	No HF
out-HF	Negative	Negative	Negative	Serogroup B	No HF	Serogroup C
100m-HF	Negative	Negative	Negative	Negative	Serogroup C	Negative

In: indoor, out: outdoor, CF: Chicken feces, HF: Housefly.

[1]Rep: Replication.

Farm 3 (Coastal)

Average *Salmonella spp., Campylobacter spp.*, and APC counts (log CFU/fly) recovered from the fly species in Farm 3 (Coastal) are shown in Table 3.9. The number of captured flies was deficient around this farm when compared the other collection rate from the two farms other farms, and this was likely due to the hurricane (Dorian) in the area after the first week of collection. A total of 192 flies created six groups, including out-HF (N = 18), 100m-HF (N = 44), out-Vesp (N = 10), 100m-Vesp (N = 34), out-DB (N = 30), and 100m-DB (N = 56) were captured in two replications. No flies were caught in the houses. Also, no HF was caught outside the houses in rep 1. Most of the collected flies were from 100 m, totaling 134 flies. The dominant insect type was the Darkling Beetle on this farm.

Average *Salmonella spp.* and APC count in out-HF and 100m-HF were almost the same for *Salmonella* (6.7 and 6.8) and APC (8.8 and 8.6) log CFU/fly.

Average *Salmonella spp.* in 100m-Vesp was about 0.4 logs higher than out-Vesp (6.5 and 6.1), and average APC plates in 100m-Vesp was slightly lower than out-Vesp (8.7 and 8.8 log CFU/fly). Out-DB and 100m-DB had almost the same range in *Salmonella spp.*, but out-DB was slightly higher than 100m-DB with the log 8.1 and 7.9. However, numbers of *Campylobacter spp*. recovered from the flies collected as treatment out-DB were nearly 2 logs higher than the number of *Campylobacter spp.* recovered from the 100m-DB flies.

Table 3.9 Fly numbers, Average *Salmonella*, *Campylobacter spp.* and Total Aerobic Bacteria (Log CFU/fly) recovered from the fly species in Farm 3 (Coastal).

Location/	Fly numbers			Salmonella Log (CFU/fly)				Campylobacter Log (CFU/fly)				Aerobic Count Log (CFU/fly)			
Fly type	Rep[1] 1	Rep[1] 2	total	Rep 1	Rep 2	Ave[2]	STD[3]	Rep 1	Rep 2	Ave	STD	Rep 1	Rep 2	Ave	STD
out-HF	10	18	18	0.0	6.7	6.7	0.0	0.0	5.7	5.7	0.0	0.0	8.8	8.8	0.0
100m-HF	27	17	44	6.7	6.8	6.8	0.1	4.2	3.6	3.9	0.4	8.7	8.5	8.6	0.1
out-Vesp	5	5	10	6.4	5.9	6.1	0.4	4.5	4.6	4.5	0.1	8.9	8.7	8.8	0.1
100m-Vesp	15	19	34	6.3	6.7	6.5	0.3	2.8	4.6	3.7	1.3	8.8	8.6	8.7	0.2
out-DB	12	18	30	7.1	6.9	7.0	0.1	4.4	4.2	4.3	0.2	8.3	7.9	8.1	0.3
100m-DB	27	29	56	7.0	6.9	6.9	0.1	0.0	4.4	2.2	3.1	8.0	7.8	7.9	0.2

Out: outdoor, HF: Housefly, Vesp: Vespidae, DB: Darkling Beetle.

[1]Rep refers to the replication recorded during the study; [2]Ave refers to the average value recorded during the study; and [3]STD refers to standard deviation.

All presumptive *Campylobacter spp.* plates in the farm were negative for *Campylobacter jejuni* in the PCR test. *Salmonella spp.* PCR and agglutination results from three farms are shown in Table 3.8. In-CF and 100m-HF in the first replication and out-HF in the second replication were determined to be *Salmonella spp.* positive. These samples were determined to contain Serogroup C *Salmonella* by the agglutination test, and all seemed identical (Figure 3.8 and 3.9). CF samples

were collected only in the second replication for this farm. Due to the interruption by the hurricane in the area, fly traps in the first replication and second replication were collected simultaneously.

Welcolex Colour Salmonella Agglutination Test.

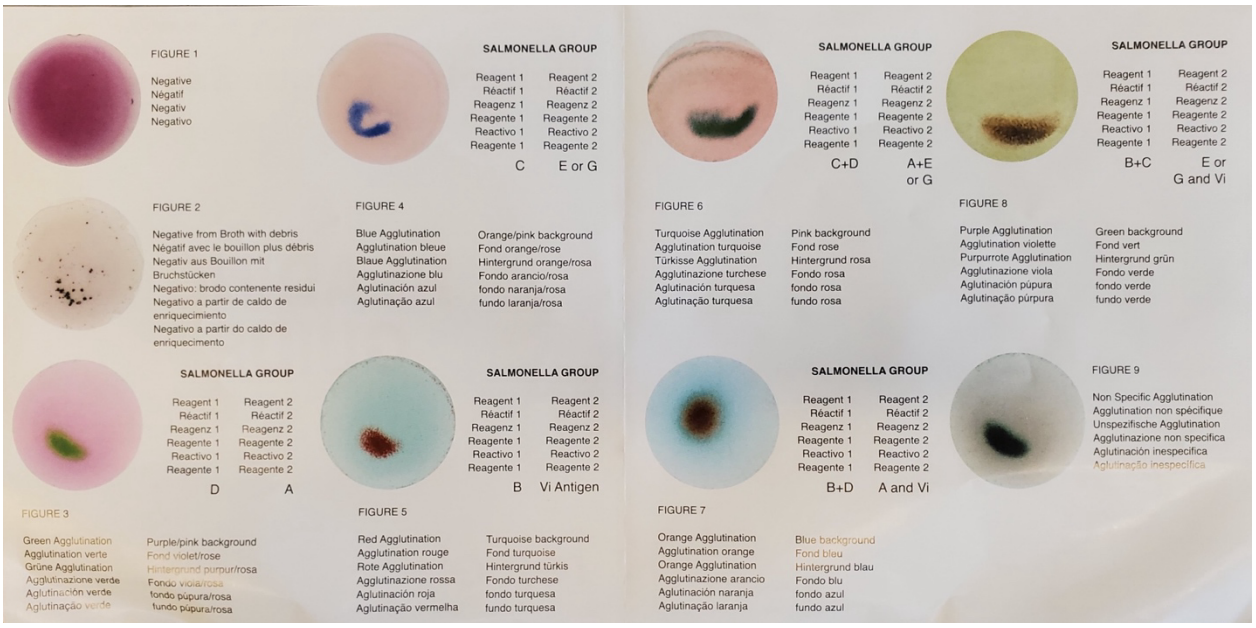

Figure 3.8 Welcolex Colour *Salmonella* Reading Guide.

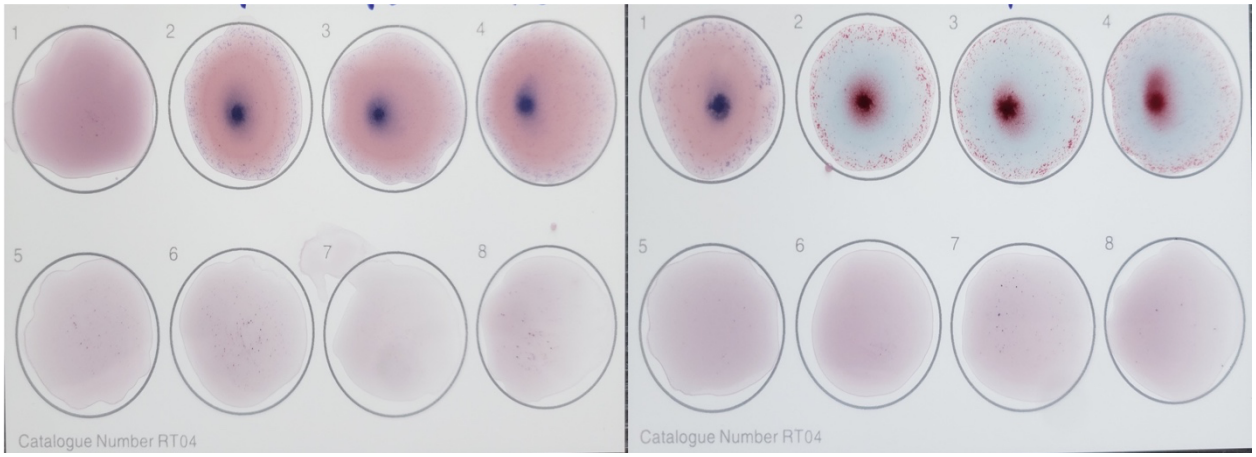

Figure 3.*9 Salmonella* agglutination test results

Two circles from top to bottom have the same samples with two different reagents. Reagent 1 was used in the first line and Reagent 2 was used in the second line from left to the right.

Samples with red agglutination with turquoise background from Midland Farm. (*Salmonella* Serogroup B).

Samples with blue agglutination with pink background are from Coastal Farm. (*Salmonella* Serogroup C).

Based on the Welcolex Colour *Salmonella* reading guide (Figure 3.8), blue agglutination with orange/pink background obtained with reagent 1 shows *Salmonella* Serogroup C. Red agglutination with turquoise background obtained with reagent 1 shows *Salmonella* Serogroup B.

Discussion

Salmonella

There are more than 2600 serovars of *Salmonella* that are currently recognized, and more than 50% of these serotypes belong to the *S. enterica* subspecies which cause illnesses in both humans and animals (Guibourdenche et al., 2010; Mezal et al., 2014). Although most *Salmonella* serotypes can grow at the range of 5-47 °C with an optimum of 32-35 °C, some few serotypes can grow at the temperatures as low as 2-4°C or as high as 54°C (Pui et al., 2011). According to the Foodborne Diseases Active Surveillance Network (FoodNet) 2021, 5,442 out of 6,110 *Salmonella* isolates (89%) were serotyped in 2021. The most common serotypes were *enteritidis* (17%) (serogroup D), *newport* (11%) (serogroup C), *typhimurium* (9%) (serogroup B), *javiana* (7%) (serogroup D), i 4,[5],12:i:- (6%) (serogroup B), *oranienburg* (5%) (serogroup C), and *infantis* (4%) (serogroup C). The five *Salmonella spp.* of *enteritidis*, *newport*, *typhimurium*, *javiana*, and i 4,[5],12:i:- have been

the most common *Salmonella* serotypes causing infection since 2010 (Collins et al., 2022). Some *Salmonella* serogroups are shown in the Table 3.10.

Table 3.10 *Salmonella* Serogroups B, C, D, and E (Fuche et al., 2016; WVDL).

Serogroup B	Serogroup C	Serogroup D	Serogroup E
Typhimurium	Newport	Enteritidis	Anatum
I 4,[5],12:i:-	Oranienburg	Javiana	Muenster
Heidelberg	Infantis	Typhi	Uganda
Saintpaul	Kentucky	Berta	London
Agona	Muenchen	Dublin	Give
Schwarzengrund	Montevideo	Panama	Senftenberg
Brandenburg	Thompson	Ouakam	Orion
Kiambu	Bareilly		Kouka
Budapest	Hadar		Liverpool
Derby	Mbandaka		
	Hartford		
	Braenderup		
	Choleraesuis		
	Tennessee		
	Manhattan		
	Bovismorbificans		

In the present study, no PCR positive *Salmonella spp*. were detected in the Farm 1. However, PCR positive *Salmonella spp.* were found on the Farm 2 and Farm 3. All positive fly groups were house flies. Other groups including wasps, flesh flies, darkling beetles and blow were found to be negative for *Salmonella spp.* Additionally, cow manure samples around the Farm 1 and Farm 2, dog feces around Farm 1 tested negative for *Salmonella spp.* (Figure 3.10).

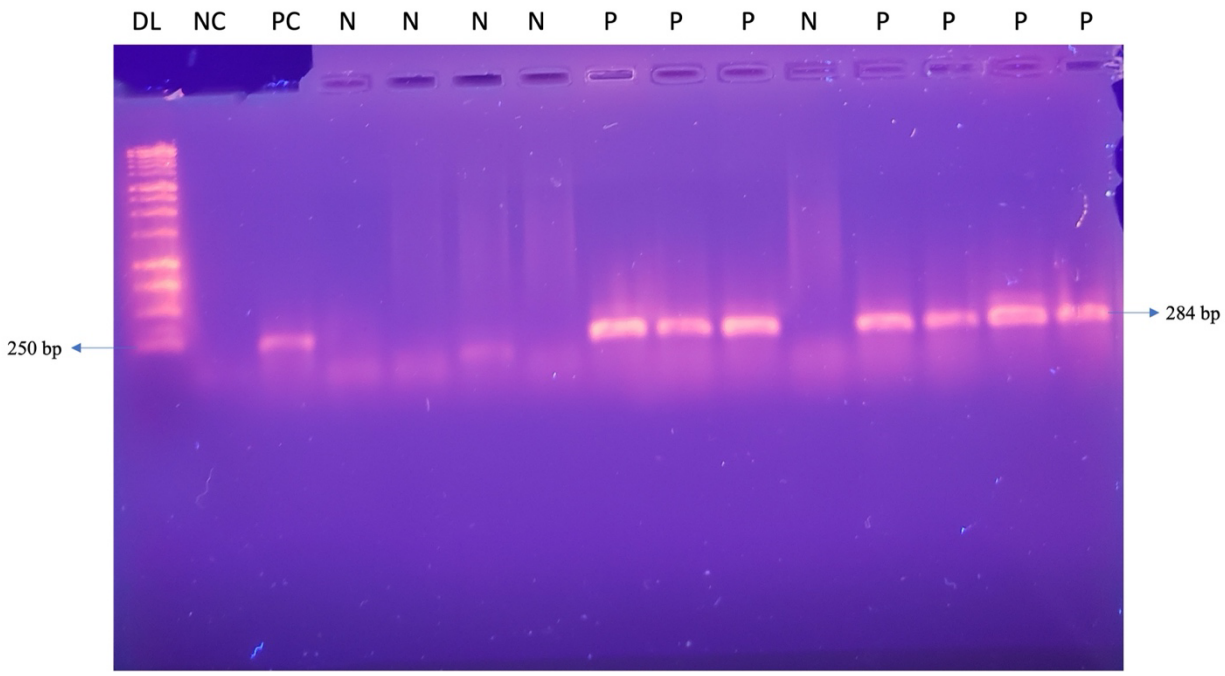

Figure 3.10 PCR amplification of invA gene of *Salmonella spp.*

PC: Positive control (*Salmonella typhimurium*)

NC: Negative Control (*Listeria*)

DL: 1 kb DNA Ladder

P: Positive samples

N: Negative samples

For farm 2 (Midland), In-CF and in-HF in both replications and out-HF in replication two were determined to be Serogroup B *Salmonella spp.* positive. They all appeared to be in the same serogroup, and no positive *Salmonella spp.* found at 100 m fly groups suggesting that *Salmonella* most likely transferred from inside to just outside of the house, not to 100 m for this specific farm. *Salmonella typhimurium* (Serogroup B) and *Salmonella enteritidis* (Serogroup D) are the prominent isolates accounting for approximately half of all human infections of nontyphoidal

Salmonella spp. globally among more than 2500 nontyphoidal *Salmonella spp.* (Tennant et al., 2016). Shivaning Karabasanavar et al. (2020) isolated *Salmonella spp.* (*S. enteritidis* (68.1) and *S. typhimurium* (31.8)) from retail market chicken meat (4.8%), live chicken on the farm (2.5%), and eggs (2.1%).

In the serogroup B, *S. typhimurium* and I 4,[5],12:i:- (a monophasic variant of *Salmonella typhimurium*) have been among the most common *Salmonella* serotypes causing infection since 2010. The EFSA and ECDC (2021) reported that *S. typhimurium* was mainly linked to pig (42%), broiler (34.8%) and layer sources (13.5%). Monophasic *S. typhimurium* was mostly associated with pig (72.1%) and next with broiler (17.1%) sources.

For farm 3 (Coastal), In-CF and 100m-HF in the first replication and out-HF in the second replication were determined to be Serogroup C *Salmonella spp.* positive, which all seemed identical suggesting that house fly most likely transferred *Salmonella spp.* from the chicken feces in the house to outdoor and 100 m distance.

In the serogroup C, *S. newport, S. oranienburg*, and *S. infantis*, were declared in the FoodNet 2021 report among the seven most common serotypes causing infection (Collins et al., 2022). Four serogroup C *Salmonella serovars (S. choleraesuis, S. tennessee, S. manhattan, and S. bovismorbificans)* are found in the group of 10 deadliest serovars isolated in the United States between 1996 and 2006. *S. dublin* is the deadliest serovar, and the only one serovar from serogroup D (Fuche et al., 2016; Jones et al., 2008).

In the EFSA and ECDC (2021) report, *S. Infantis* (serogroup C) mostly was found related to broiler sources (93.1%).

Dunn et al., (2022) studied *Salmonella* prevalence in poultry litter from thirteen farms across four southern states in the US. Six (46.2%) farms tested *Salmonella* positive. The prevalence of

Salmonella reported 33 (6.7%) out of 490 samples collected from 70 piles (dry stacks) or the ground (poultry houses and pastures). From among the six states tested, farms in Georgia (54.5%, 18/33) had the highest *Salmonella* positive rate, followed by Texas (30.3%, 10/33), Alabama (12.1%, 4/33), and Florida (3.0%, 1/33). They found that *S. kentucky* (serogroup C) (45.5%), *S. kiambu* (serogroup B) (18.2%), and *S. michigan* (12.1%) were the most prevalent serotypes on these farms (Dunn et al., 2022). *S. kentucky* was found on 4 of the 6 positive farms with the highest distribution among the farms. They also found that *Salmonella spp.* was more likely to be captured from the poultry house floor and pasture than stacked litter piles, and they stated that the likelihood of *Salmonella spp*. recovery decreased as the litter age increased.

Previously, the same specific *Salmonella newport* (serogroup C) was involved in the outbreaks of salmonellosis from contaminated tomatoes in the Eastern Shore of Virginia (Bell et al., 2015; Bennett et al., 2015; Greene et al., 2008), and cucumbers originated from the Eastern Shore of Maryland (Angelo et al., 2015). Bell et al. (2015) suggested *Salmonella newport* may have the potential to persist in the agricultural and natural environments. Gu et al., (2019) found *Salmonella spp*. in different water sources (creek, well, bay), broiler farms (raw poultry litter), and poultry litter amended soils (in agricultural fields) and reported that *Salmonella newport* (serogroup C) was the predominant serovar isolated from water samples. In contrast, a significantly lower percentage of *S. newport* was isolated in raw poultry litter, and poultry litter-amended field soils (Gu et al., 2019). *Salmonella* typhimurium (serogroup B), kentucky (serogroup C), and thompson (serogroup C) were the prominent serovars in raw poultry litter, and poultry litter amended field soils (Gu et al., 2019).

Salmonella outbreak in a poultry flock may result from environmental sources, such as rodents, wild birds, feed, insects, transportation, and farm environment. The hatchery can also be an important source of *Salmonella* contamination in a poultry operation.

Bailey et al. (2001) recovered *Salmonella spp.* from 26 samples collected from various locations throughout a hatchery to the end of slaughter on 32 integrated broiler farm operation. Most prevalent *Salmonella* sources were hatchery transport pads (50.8%), flies (18.7%), drag swabs (14.2%), and boot swabs (12%).

In addition to the mentioned sources, poultry feed may also be a source of contamination (Jarquin et al., 2009). Jajere et al. (2019) reported that *Salmonella* was detected in 5.14% (9/175) of drinking water, 7.14% (5/70) of poultry feed, and 5.0% (3/60) from fly samples collected from 35 chicken flocks in Malaysia. Bucher et al. (2007) reported *Salmonella spp.* from broiler feed and the packaged raw, frozen chicken nuggets and strips were indistinguishable.

Campylobacter

C. jejuni, C. coli, and *C. lari* are three common species of *Campylobacter* in the poultry gastrointestinal track system. *C. jejuni* is the most common type affecting human health, and to a lesser extent, *C. coli,* and *C. lari*, *C. upsaliensis*, *C. fetus* types affect human health as well (Cean et al., 2015; Perez-Arnedo & Gonzalez-Fandos, 2019; Shane, 1992; Ugarte-Ruiz et al., 2018; Wagenaar et al., 2013).

High levels of *Campylobacter spp.* (mainly *C. jejuni* and *C. coli*) are found as a natural component of the gastrointestinal tract of commercial chickens, without causing disease in the chickens (Corry & Atabay, 2001; Sahin et al., 2002, 2015). Previous studies reported that the prevalence of *Campylobacter spp.* positive poultry flocks might range from 2% to 100% depending on regions,

seasons (environmental temperature and rainfall), and production types (conventional, free-range, and organic) (Sahin et al., 2015). In this study, only *C. jejuni* was tested in the PCR for all samples. Also, *C. coli* was tested in all samples collected from Farm 2 (Midlands), along with cow manures collected 400-500 m away from the houses. All fly groups and chickens' feces from three commercial broiler farms were found *to be C. jejuni* free (Figure 3.11).

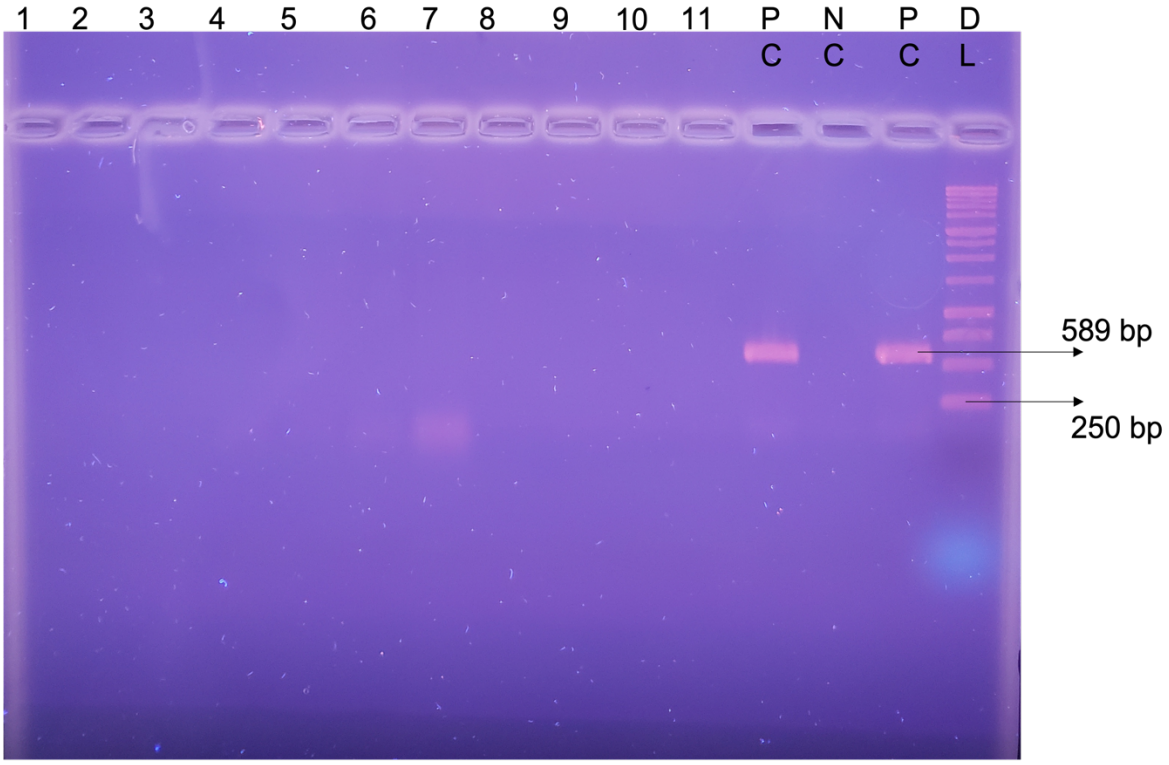

Figure 3.11 PCR amplification of mapA gene of *Campylobacter jejuni.*

PC: Positive control (*Campylobacter jejuni 33560* ATCC).

NC: Negative Control (*Micrococcus luteus*).

DL: 1 kb DNA Ladder.

Numbers 1-11: Negative samples.

Campylobacter spp. require a microaerophilic environment containing 5% oxygen, 10% carbon dioxide, and 85% nitrogen and the temperature of 31–44 ^{0}C with optimum 42 ^{0}C (Shane, 1992; Stanley et al., 1998). In the present study, indoor max temperatures in the chicken house were 30 ^{0}C and indoor average temperatures were less then 30 ^{0}C for Farm 1 and 3. For Farm 2, average indoor temperature is 30.8 ^{0}C, and max indoor temperature is 34 ^{0}C. These temperatures are not as favorable for *Campylobacter spp.* to grow. Outside temperatures reached to max 34 ^{0}C for the farm three and 36 the other farms. Average outside temperature 23.5, 26.3, and 25.6 ^{0}C for the farm 1, 2, and 3, respectively.

Positive *C. coli* were found in cow manure, out-HF, 100m-Callip, and 100m-DB in farm 2. This indicates that the *C. coli* source was from outside the poultry house rather than from inside the houses. Royden et al. (2016) detected a low prevalence of *Campylobacter spp.* from flies on three broiler farms in the UK. They found 0.22% (2/902) from individual flies and 3.15% (4/127) from grouped flies by family carried *Campylobacter spp*. Hald et al. (2008) found 31 out of 2816 (1.1%) flies captured around the five Danish broiler farms were positive for *Campylobacter spp.* (7 *C. jejuni,* 23 *C. coli,* and 1 other *Campylobacter spp.).* House flies were found to carry *Campylobacter spp*. more frequently, but only 1 of 488 Calliphoridae carried *Campylobacter spp.* These same authors also reported that the prevalence of *Campylobacter spp*. changed from 0% in April to maximum 16.3% in July, and 2% in October on one farm with swine production, while the prevalence was continually below 1% on the rest four broiler farms without other livestock during the study (April to November).

The prevalence of *Campylobacter spp*. is usually uncommon in young broiler chickens less than 2-3 weeks of age under commercial production conditions and increases as the birds grow and it reaches maximum prevalence (close to 100% in some infected flocks) in broilers at the slaughter

age (Sahin et al., 2015). In the current study, chicks were 1-2 weeks old when the first traps were set thus, it was expected that the *Campylobacter* counts would be lower than if the flock were slaughter age.

Conclusion

House flies were found to transmit *Salmonella spp.* 100 m away from broiler farms. This supports the theory that flies transmit these pathogens in and around broiler houses. Hald et al. (2007) showed that fly screens decreased *Campylobacter spp*. infections from 51.4% to 15.4% in 20 broiler houses during the summer by means of preventing the influx of flies in the houses. In the current study, *Campylobacter coli* positive samples were verified in cow manure samples (400-500 m away from the houses), 100m-Calliphoridae, out-HF, and 100m-DB on farm 2, suggesting that Calliphoridae, house flies, and Darkling Beetles are a vector in transmitting *C. coli* on farms. The primary fly type found both inside and outside broiler houses was the house fly, and thus, these insects may be more active in transferring pathogens around poultry houses rather than other fly types. Even though wasps were commonly captured on all three farms, they were not found to carry *Campylobacter spp.* or *Salmonella spp.*

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CHAPTER FOUR

CONCLUSION

The first part of the research involved controlled laboratory experiments to determine the short time exposure effects on bacterial transfer by fruit flies (wingless fruit flies, *Drosophila melanogaster* Meigen) from and to food. Initially, short-time exposure (1, 5, and 15 min) of flies to inoculated apple slices was tested to determine the transfer of *E. coli* to flies. Then, the transfer of *E. coli* from inoculated apple or bologna slices (5 min exposure) to un-inoculated slices (1, 5, and 15 min exposure) was tested. The percentage of *E. coli* transferred from inoculated food to flies was low (<0.5%), while the rate transferred from flies to un-inoculated food was relatively high (>50%). This study concluded that flies could pick up and transfer bacteria to food in short exposure times.

It would be ideal to have control samples of apples and bologna that were exposed to *E. coli* for short periods (1, 5, and 15 minutes) so we can answer some unanswered questions. For example, short time exposure of flies to contaminated food and subsequent short time exposure to uncontaminated food results in transfer of relatively high numbers of bacteria baloney has more transfer than apple, but did the food's pH or composition affect this? Also, did the number of *E. coli* bacteria on the food increase or decrease during the exposure time?

Apples are composed primarily of carbohydrates and water. A medium-sized apple (100 grams) that is raw and unpeeled contains the following: water makes up 86% of it, protein is 0.3 grams, carbohydrates are 13.8 grams, fiber is 2.4 grams, and fat is 0.2 grams (Arnarson, 2023).

However, Turkey bologna is primarily composed of water, protein, and fat. 100 grams of bologna consists of 4.4 grams of carbohydrates, 11.4 grams of protein, and 17.8 grams of fat (https://nutrientoptimiser.com/nutritional-value-bologna-turkey/). The availability of

nutrients in food can influence the growth of *E. coli* populations. For example, *E. coli* grows in foods that are high in protein and bologna has higher protein than apple. Moreover, the pH level of food can also affect the growth of *E. coli.* *E. coli* can grow in foods with a pH between 4.6 and 9.0 (De Jonge et al., 2003), but it grows best in foods with a neutral pH of around 7.0 (Davey, 1994). Apples have a low pH level of 3.6, which creates an environment that is unfavorable for E. coli growth and survival. Apples also have natural compounds, such as polyphenols and flavonoids, which have antimicrobial properties and can inhibit the growth of bacteria, including E. coli. Bologna has a slightly acidic pH level of 4.5, which can slow down the growth rate of E. coli but may be more favorable for E. coli growth than apples because it is not as acidic.

The second part of the research involved field experiments to examine the migration of pathogens, such as *Salmonella* and *Campylobacter spp.,* via flying insects within a 100 m range of commercial poultry farms. House flies were found to transmit *Salmonella spp.* 100 m away from broiler farms suggests that flies could play a role in spreading these pathogens in and around broiler houses. Since house flies were the most prevalent fly species inside and outside broiler houses in this study may be more active than other fly species in spreading pathogens surrounding poultry houses. *Campylobacter coli*-positive samples were found in cow manure samples (400-500 m away from the houses), 100m-Calliphoridae, out-HF, and 100m-DB on a farm; thus, Calliphoridae, house flies, and Darkling Beetles might be a vector in the transmission of *C. coli* on some farms. Vespidae fly groups were commonly captured on all three farms. However, they were not responsible for spreading *Campylobacter spp.* or *Salmonella spp.*.

It would be better if all three poultry farms in the experiment had the same conditions, either with a nearby cattle farm or without one. Farm 1(Upstate) had no other farms around it, but farms 2 (Midland) and farm 3 (Coastal) had a small cattle herd nearby.

The presence of a cattle herd near broiler farms can increase the risk of bacteria transmission between the two species. Cattle can carry *Salmonella spp., Campylobacter spp.,* and *E. coli spp.* in their digestive tract, and the bacteria can be shed in their manure. Since flies are attracted to manure, they can pick up these bacteria on their bodies and transfer them to other surfaces, including the broiler farm environment, feed, water, and even broiler chickens themselves. Moreover, flies can travel long distances, and it is therefore necessary to keep a considerable distance between the cattle and broiler farms to minimize the risk of disease transmission.

Removing manure and other organic materials from the broiler farm environment and using screens or other physical barriers to prevent flies from entering the broiler farm would be helpful to prevent the transmission of bacteria by flies.

Also, to isolate *Campylobacter,* we used the *Campylobacter* media (mCCDA) with a supplement. However, during our experiment, we discovered that the control samples of *Campylobacter jejuni* did not grow on this particular media with the supplement, but they did grow on the media without the supplement. Therefore, using both the media with and without the supplement would be great during the isolation process.

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APPENDICES

Appendix A

Insects and their numbers/locations collected from Farm 1 (Upstate)

Farm 1 (Upstate) - Outdoor

house #	Rep 1 out-HF	Rep 2 out-HF	Rep 1 out-Vesp		Rep 2 out-Vesp		Rep 1 out-Sarcop	Rep 2 out-Sarcop
			Yellow Jacket	Paper Wasp	Yellow Jacket	Paper Wasp		
1	25	24	1	0	0		0	6
2	26	48	1	0	3	0	0	3
3	64	48	3	0	3	0	0	4
Total	115	120	5	0	6	0	0	13

Farm 1 (Upstate) - 100 m

Location	HF	Vesp	Sarcoph
Rep 1		**Yellow jacket**	
North	18	3	0
West	26	0	0
South	51	0	0
East	33	3	0
Total	128	6	0
Rep2			
North-East	12	1	0
North-West	9	4	6
South-East	80	5	0
South-West	29	5	3
Total	130	15	9

Appendix B

Insects and their numbers/locations collected from Farm 2 (Midlands)

Farm 2 (Midland) - Indoor

house #	Rep 1	Rep 2
	in-HF	in-HF
1	6	12
2	55	27
3	12	2
4	4	3
5	17	15
6	50	20
7	9	4
8	6	47
total	159	130

Farm 2 (Midland) - Outdoor

house #	Rep 1	Rep 2	Rep 1		Rep 2	
	out-HF	out-HF	out-Vesp		out-Vesp	
			Yellow Jacket	Paper Wasp	Yellow Jacket	Paper Wasp
4	213	114	24	0	13	1
5	290	153	5	0	7	0
Total	503	267	29	0	20	1

Farm 2 (Midland) - 100 m

Location	HF	Vesp	Vesp
Rep 1		Yellow Jacket	Paper Wasp
North	53	9	7
West	30	6	3
South	37	4	1
East	40	19	0
Total	160	38	11
Rep2			
North-East	20	6	0
North-West	12	2	3
South-East	44	6	3
South-West	9	2	0
Total	85	16	6

Appendix C

Insects and their numbers/locations collected from Farm 3 (Coastal)

Farm 3 (Coastal) - Indoor

house #	Rep 1	Rep 2
	in-HF	in-HF
1	0	0
2	0	0
3	6	3
4	0	2

Farm 3 (Coastal) - Outdoor

house #	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
	out-HF	out-HF	out-Vesp	out-Vesp	out-DB	out-DB
			Yellow Jacket	Yellow Jacket		
2	5	12	2	1	5	6
3	5	6	3	4	7	12
Total	10	18	5	5	12	18

Farm 3 (Coastal)-100 m

Location	HF	Vesp	Vesp	DB
Rep 1		Yellow Jacket	Paper Wasp	
North	1	5	0	2
West	15	2	0	0
South	6	2	0	22
East	5	6	0	3
Total	27	15	0	27
Rep2				
North-East	5	0	8	3
North-West	7	3	0	0
South-East	2	0	7	2
South-West	3	0	1	24
Total	17	3	16	29