Effect of biosecurity and management practices on the prevalence of Salmonella, Campylobacter, and Clostridium perfringens in a poultry production system

by

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Abstract

Salmonella, Clostridium perfringens, and *Campylobacter* are responsible for 30% of all foodborne illness cases and 50% of foodborne illness hospitalizations in the United States. Many of these cases and outbreaks have been linked to poultry products as the source of contamination. Because of the significant impact these pathogens have on human health, they are direct targets for pathogen reduction programs at various points during poultry production. Pre-harvest control points are the most recent targets for pathogen control due to increasing costs, regulations and the presence of diseases such as Avian Influenza (AI) and Necrotic Enteritis (NE) caused by *C. perfringens.* Of particular interest are the specific biosecurity and management practices that influence the transmission of pathogens in the poultry production environment. This study aimed to determine which practices influenced the transmission of *Salmonella, C. perfringens,* and *Campylobacter* on commercial poultry farms. In addition, this study collected data on the presence of two novel *C. perfringens* genes (*netB* and *tpeL*) that produce toxins and are associated with the disease NE in poultry.

A biosecurity and management survey was sent out to all of the growers for a single Integrator in Alabama. Responses were collected, analyzed and general recommendations for biosecurity and management program improvement were made to Integrator management. A year later, the survey was sent to the same growers to determine if there were any differences in practices. The reported farm characteristics for both surveys were very similar with only two instances of statistical significance. There was no statistical significance between the two surveys

for any biosecurity or management practice. These results in conjunction with conversations with the growers indicated that the company did not make any changes to their biosecurity and management program as a result of our recommendations. In addition, compliance with many of the most common biosecurity practices was better than that found by previous studies (Dorea et al., 2010; Vieira et al., 2009; Tablante et al., 2002).

After the first survey was sent to the growers and the responses analyzed, four farms from the same Integrator were selected based on the average characteristics indicated by the survey. These four farms were sampled three times at even intervals throughout one grow-out period. During each visit, drag swabs, cloacal swabs and litter samples were obtained from each house and then analyzed for the presence of *Salmonella, Clostridium perfringens,* and *Campylobacter*. The total anaerobic and aerobic bacteria counts were also obtained from the litter samples. After the second survey was sent to the growers, the same four farms were again sampled in the same manner as before to determine if any differences in the microbial community could be detected as a result of changes in biosecurity and management practices. Only one instance of statistical significance ($p<0.05$) was found for all the samples tested for the presence of *Salmonella.* Recovery of *Campylobacter* was poor for the first sampling due to the cultivation methods used. After the recovery methods were adjusted, this organism was recovered at much higher levels using cloacal swabs. *Campylobacter* was not recovered at any point during this study from the drag swab samples. Only two visits (cloacal swabs) were statistically significant ($p<0.05$) for this organism. Recovery of *C. perfringens* was statistically significant ($p<0.05$) for four of the six visits. The anaerobic and aerobic bacteria each had three visits that were significantly different ($p<0.05$). PCR results for the two novel *C. perfringens* genes were consistent with previous findings and contribute to the growing body of evidence that suggests that *tpeL* is not as

important of a virulent factor as previously thought and that while *netB* is an important virulent factor it is inconsistently present in *C. perfringens* isolates.

The results of this study were unable to directly identify which specific biosecurity and management practices influenced bacterial prevalence on the commercial poultry farm. Despite this fact, valuable information was collected regarding common on-farm biosecurity and management practices, their compliance, and the microbial populations taken from a poultry house environment.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AI	Avian Influenza
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CDC	Centers for Disease Control and Prevention
FDA	Federal Department of Agriculture
FDOSS	Foodborne Disease Outbreak Surveillance System
HACCP	Hazard Analysis and Critical Control Point
LEDS	The Laboratory-based Enteric Disease Surveillance
NARMS	National Antimicrobial Resistance Monitoring System
NE	Necrotic Enteritis
NNDSS	National Notifiable Disease Surveillance System
NPIP	National Poultry Improvement Plan
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PPIA	Poultry Products Inspection Act
SAP	Salmonella Action Plan
SIDS	Sudden Infant Death Syndrome

TSA II	Trypticase Soy Agar with 5% Sheep Blood
TSC	Tryptose Sulfite Cycloserine
TTB	Tetrathionate Brilliant Green Bile Broth

List of Abbreviations (continued)

USDA	United States Department of Agriculture
USDA-APHIS	USDA Animal and Plant Health Inspection System
USDA-FSIS	USDA Food Safety Inspection Service
XLT4	Xylose Lysine Tergitol 4

I. Introduction

According to the Centers for Disease Control and Prevention, there are approximately 48 million cases of foodborne illness per year in the United States, 3,000 of these cases resulting in death. *Salmonella, Clostridium perfringens* and *Campylobacter* together account for 30% (over 2.8 million illnesses) of foodborne illness cases acquired domestically. In addition, *Salmonella* and *Campylobacter* together are responsible for 50% of foodborne illness hospitalizations (Centers for Disease Control and Prevention [CDC], 2012; CDC, 2011). Improperly handled, contaminated poultry meat and eggs cause many of these cases. *Salmonella spp.* and *C. perfringens* are also a concern in poultry production, causing decreased efficiency, disease and mortality. For these reasons, and their associated economic significance, controlling these pathogens and decreasing their exposure to production animals and as a result, the consumer, is of the utmost importance.

Historically, pathogen control efforts in the poultry industry have been focused on antibiotic use during production, and post-harvest strategies to reduce the existing pathogen load. Increasing costs and regulations combined with the domestic presence of diseases such as Avian Influenza (AI) have encouraged the industry to explore alternative control methods. Pre-harvest points have recently been targeted as an optimal point for pathogen control and reduction. Of particular interest are the methods by which pathogens spread horizontally, from farm to farm, bird to bird, and what factors contribute to their spread. Once this is known, control efforts can be targeted at the points in production that would have the most impact.

II. Literature Review

The purpose of this review is to determine how *Salmonella*, *Campylobacter,* and *Clostridium perfringens* spread within a poultry production system, and to determine what factors contribute to their occurrence. In this study, the management and biosecurity practices of poultry farmers were examined and samples were taken from the poultry house environment. The bacterial isolates obtained during this study were evaluated using classic microbiological techniques. Both *Salmonella* and *C. perfringens* can commonly be found in the environment and can survive there for long periods of time (Mueller-Spitz, 2010). These bacterial species in addition to *Campylobacter* have very distinct and separate disease and growth characteristics and therefore must be addressed separately.

Salmonella

History of Salmonellosis

Theobald Smith discovered the first member of the genus *Salmonella* in 1885. *Salmonella enterica*, also known as *S. choleraesuis* was isolated from porcine samples in an effort to identify the cause of hog cholera (Schultz, 2008). The *Salmonella* genus, which is also a member of the family *Enterobacteriaceae* is split into two species, *S. enterica* and *S. bongori*, both of which can cause disease in humans. *S. enterica* is the most significant in public health terms, and is divided into six subspecies, *S. enterica* subspecies *enterica* (I), *S. enterica* spp. *salamae* (II), *S. enterica* spp. *arizonae* (IIIa), *S. enterica* spp. *diarizonae* (IIIb), *S. enterica* spp. *houtenae* (IV), and *S. enterica* spp. *indica* (VI) (Salmonella species; Adair et al, 2008) Each subspecies of *Salmonella* is further divided into serotypes based on the slide agglutination test from the 1930s and modified by Kauffmann-White-Le Minor (Wattiau, 2011). This test distinguishes different *Salmonella* serotypes based on their expressed surface and flagellar

antigens (Schultz, 2008). To date, there have been over 2,500 serotypes, many of which are of epidemiologically importance (Adair et al, 2008). *Salmonella* has been isolated from every type of food animal, causing morbidity and mortality in the infected animals as well as passing on the infection to human consumers. *Salmonella* has evolved to be host specific, and many serotypes are associated with specific host species, whereas other types can cause disease or be carried by multiple species (Callaway, 2007; World Health Organization [WHO], 2013). Anyone can become sick from the ingestion of an infective dose from contaminated food but the young, elderly and immune-compromised can more easily contract the disease (Acheson, 2001).

Poultry and their products have long been associated with *Salmonella* infections. However, as one study suggests, the serotypes recovered from human samples can be different than those carried by food animals. This can have several implications; first, the ability of different *Salmonella* serotypes to cause human disease can vary, and second, some serotypes are limited to either animal or human populations, or that they may be spread more favorably in one population. These implications may indicate that the assumption that food animals are usually the source for human Salmonellosis may be slightly biased (Sarwari, 2001).

Salmonella is a gram negative, straight, non-spore forming rod that is a facultative anaerobe. There are motile and non-motile forms that can be found in contaminated water or food, and are members of the natural gut micro flora of most animals. *Salmonella* is a facultative intercellular pathogen that produces an endotoxin, usually a lipopolysaccharide, which is released into the host's bloodstream when the bacterium is lysed. This pathogen can also cause disease by producing enterotoxins that target intestinal cells (Ashkenazi, 1988; Adair et al, 2008).

Over 42,000 cases of salmonellosis are reported every year. However according to the CDC, they estimate that there are over a million cases of salmonellosis every year. Since many mild forms of the disease go unreported, this number of reported illnesses is vastly underestimated (What is Salmonellosis, 2015; Olsen, 2001). The economic cost for *Salmonella* infections is approximately $3.67 billion per year in the United States (Cost Estimates, 2014). Additionally, it has been estimated that 95% of *Salmonella* infections are foodborne based (Acheson, 2001). In 2014 there were ten official *Salmonella* outbreaks, two were associated with poultry and their products. *Salmonella* Heidelburg, Infantis, Newport, and Hadar were the etiological agents of the outbreaks associated with poultry. The first outbreak was linked to flocks of backyard poultry, had 363 cases with 120 hospitalizations and covered 43 states. The second outbreak was traced to a poultry meat product, and featured two isolates that were multi-drug resistant. This outbreak had 9 cases with a 22% hospitalization rate and covered one state (Reports of Salmonella, 2015).

Salmonella infections harm not only the consumer, they also negatively impact the companies responsible for producing the contaminated product due to recalls, lawsuits and the negative image consumers may have towards that company and product. In 2010 the largest recorded outbreak of *S.* Enteriti*s* occurred. More than 2,500 people became ill and over 500 million shell eggs were recalled. Media coverage and the negative image they portrayed caused egg prices to plummet and the egg industry to lose $100 million in a single month. Another outbreak associated with peanut butter in 2007 cost the responsible company $78 million and the industry $1 billion (The Association of Food, Beverage and Consumer Products Companies [GMA], 2011).

From both a production and consumer standpoint, these statistics demonstrate the importance of controlling *Salmonella* infections to increase public safety and decrease economic losses.

Serotypes of Importance

The serotype distribution of *Salmonella* is constantly changing due to the strains used in vaccines, genetic changes in the *Salmonella* bacteria, increased production and consumption of certain food products such as poultry (Jackson, 2013; Foley, 2011; Olsen, 2001). However, according to one study, management practices such as the use of antibiotics or pre/probiotics have no effect on serotype distribution (Foley, 2011). One study linked the five serotypes most commonly associated with human Salmonellosis to broilers and ground chicken meat from flocks that did not show signs of infection. This indicates that food animals that silently carry this disease are more likely to enter the food supply and cause human disease than those that exhibit symptoms of disease (Salmonellosis, 2012; Callaway, 2007).

In the late 1800s to the mid-1900s the predominant serotype of public health interest was *Salmonella enterica* serotype Typhi (Olsen, 2001). *S.* Typhi is host specific, causing paratyphoid fever and only infecting and spread by humans. This bacterium grows in the intestinal tract and the blood of an infected or carrier host. *S.* Typhi is spread through feces and can be contracted by the consumption of food or beverages that have been touched by a person shedding the bacteria or having been in contact with contaminated sewage. Typhoid fever is a life-threatening illness characterized by a high fever, weakness, a rash, and headache. This type of infection is predominantly seen in underdeveloped countries and causes disease in 21.5 million people a year worldwide. The occurrence of *S.* Typhi infections has greatly decreased in the United States

thanks to sanitation protocols and vaccination programs for travelers. However, about 5,700 cases are seen each year, mostly in those that have travelled abroad (Typhoid Fever, 2013).

Fowl Typhoid and Pullorum Disease caused by *S.* Gallinarum and *S.* Pullorum respectively, are historically significant as these diseases previously caused widespread flock infection, and while it was very rare for a human to contract the disease, the economic cost due to increased flock mortality and decreased production made it economically significant (Fowl Typhoid, 2012). Previously, the etiological agent of these two diseases was classified into two separate species*, S. Gallinarum* and *S. Pullorum* respectively, but due to many similarities between the two diseases, they are now classified under the single specie aforementioned. These diseases are host specific and can be vertically or horizontally transmitted. Pullorum Disease previously known as bacillary white diarrhea mainly infects chicks and poults with mortality of up to 100% in infected flocks. Symptoms include a low hatch rate, poor growth, labored breathing and chalky excreta around the vent (Fowl Typhoid, 2009). The last reported incidence of a Pullorum Disease outbreak in the United States was in 1990. Fowl Typhoid, a disease similar to Pullorum disease was first identified in 1888 and can cause mortality of up to 60% in infected flocks. Symptoms are similar to Pullorum Disease; however this disease mainly targets adult chickens and turkeys (Adair et al, 2008). The National Poultry Improvement Plan was created in the 1930s in an effort to control Fowl Typhoid and Pullorum Disease. Under this program participant flocks are annually tested for the presence of these diseases and any contaminated flocks must be treated or eliminated (Adair et al, 2008; National Poultry, 2014)

Salmonella Enteritidis, one of the most prevalent *Salmonella* serotypes, causes foodborne illness in humans. Rising to importance in the 1980s, this illness is commonly linked to contaminated eggs that are consumed raw or undercooked. Symptoms often include diarrhea,

abdominal cramps and fever, lasting for up to a week. Laying hens silently carry this organism in their ovaries and pass it on in their eggs. It is estimated that 1 in 20,000 eggs are contaminated. Methods of control include but aren't limited to, management practices that reduce the occurrence of *S.* Enteritidis, pasteurization of eggs known to be contaminated, and fully cooking eggs and poultry meat products prior to consumption (National Center, 2010; Guard-Petter, 2001). One study suggests that the reduction in the occurrence of *S.* Gallinarum-Pullorum has opened a niche that *S.* Enteritidis was able to fill. This can also suggest that using competitive bacteria, such as inactivated *S.* Gallinarum could control *S.* Enteritidis, affecting the organism's ability to survive (Guard-Pette, 2001; Callaway, 2007).

Vigilant testing, consumer education and wide spread flock vaccination where applicable for *Salmonella* spp. has greatly decreased the occurrence of many types of Salmonellosis. However, the recent appearance of antibiotic resistant strains due to widespread antibiotic use and the fact that the pathogen is very adaptable and environmentally present makes this pathogen a major public health concern.

Factors Affecting Spread

Salmonella is naturally present in the environment and is transmitted vertically or horizontally in a poultry production system. Vertical transmission occurs when the hen carries *Salmonella* in her ovaries and, during egg formation the organism is deposited within the egg. Chicks from these contaminated eggs can then horizontally pass on the disease within the flock, which if not caught and treated could then reach consumers (Hafez, 2000). When chicks hatch the complexity of their natural gut micro flora is minimal and as such can easily be influenced by bacteria such as *Salmonella.* The gut microbial content grows and multiplies during the first two weeks and during this time colonization of the gut by *Salmonella* may occur (Crhanova, 2011).

However, the development of a mature gut micro flora population can impede the colonization of *Salmonella* due to its competition against already well-established bacterial colonies (Revolledo, 2006). This is a problem in broilers since they are harvested prior to reaching maturity and their gut micro flora does not have the opportunity to fully develop. Thus they are more prone to be colonized by *Salmonella* than mature birds.

Prior to hatching, previously uncontaminated eggs can become contaminated in the hatchery if infected eggs explode, the clean eggs are exposed to contaminated fecal material or if contaminated fluff from previous hatches come into contact with the unhatched egg or chick (Buck, 2004; Cox, 2000). Additionally, it is not unusual for eggs from several flocks to be placed in the same hatcher; if any of these eggs are contaminated they can spread the disease to previously uninfected eggs (McCrea, 2005).

Salmonella survives reasonably well in water, surviving up to 56 days in a freshwater environment. Water can become contaminated through agricultural runoff, fecal contamination, or if the water equipment was not cleaned properly after a cleanout from an infected flock (Murray, 2000).

It is well known that in the past, poultry feed was a common source of *Salmonella* in poultry (Maciorowski, 2004). The use of contaminated feed can directly infect the birds that ingest it as well as cause the water supply within the poultry house to be contaminated, spreading disease through the whole house (McCrea 2005). Feed can become contaminated through contaminated ingredients or processing equipment, and the nutrient rich environment encourages the organism's growth.

Proper biosecurity measures are very important in preventing *Salmonella* contamination within a poultry production system. Farm visitors, rodents, insects and wild animals can

introduce pathogens into the flock if they are not controlled. Additionally, if farm workers do not practice proper sanitation techniques, they can track the organism into or out of the farm, and between poultry houses. One study determined that up to 33% of wild birds found near poultry houses are contaminated with *Salmonella*, and as such could be reservoirs of possible contamination and spread (Craven, 2000).

Salmonella is a poor competitor, and while it is often present in the natural gut micro flora of poultry, its ability to survive and flourish in the litter environment is not as capable as many other pathogens. However, if proper litter management techniques are not performed, especially after the introduction of a *Salmonella* positive flock, litter can be a source of contamination for future flocks (Chen, 2014).

Methods of Control and Treatment

Since there are so many factors that can affect the spread of *Salmonella*, it is essential to properly address each factor in order to control the prevalence of *Salmonella* in the poultry production environment. One of the most important control points is to make sure that the parent flock and hatchery environment are not contaminated with *Salmonella* based on repeated testing (Mcllroy, 1989; Edel, 1994; Cox, 1990).

Maintaining a healthy flock and properly cleaning out and disinfecting the houses between flocks are important to prevent the introduction of pathogens to subsequent flocks. Previously, it was though that treating litter with an acidifying product could decrease the amount of *Salmonella* present in the litter and decrease the possibility of horizontal transmission, especially between flocks (Vicente, 2007). However, recent research has indicated otherwise. The pH reduction in the litter as a result of the acidifying product causes an increase in the amount of *Salmonella* present due to its ability to adapt and flourish under acidic conditions

(Williams, 2012). Other techniques includes adding fresh litter after each flock, completely replacing the litter and properly maintaining water lines to reduce the water activity in the litter. Windrow composting of the litter is commonly performed to reduce the pathogen load in poultry litter (Wilkinson, 2011; Macklin, 2006). It should be noted that *Salmonella* is able to survive better on new litter than old, used litter (Chen, 2014).

Using ingredients that are certified free from *Salmonella* greatly reduce the likelihood that the finished product will be contaminated with pathogens (Jones, 2011). The addition of a heat treatment or various feed additives such as probiotics, prebiotics, fatty acids, and organic acids effectively reduce *Salmonella* numbers in the normal diet (Jones, 2011; Van Immerseel, 2002; Van Immerseel, 2005). Due to the issue of antibiotic resistance, antibiotics are quickly being banned from routine use in animal feeds and as such should not be relied on as the sole source for pathogen reduction.

It is important to always use a clean, fresh water source that is free from organic and inorganic contaminants, or that the proper treatment is applied to it prior to its exposure to the birds (Byrd, 2001; Amaral, 2004). Making sure that proper biosecurity and sanitation procedures are followed will reduce the possibility of *Salmonella* contamination. Sanitizing water lines, keeping wild birds and other animals out of the houses, reducing farm visitors to necessary personnel, the routine use and maintenance of foot baths, either the use of shoe covers or dedicated shoes, rodent and insect control programs are all common biosecurity practices performed on poultry farms (Tablante, 2002; Dorea, 2010; Henzler, 1992; Jones, 1991). Several studies have shown that having inadequate biosecurity practices impacts the incidence of disease and flock performance (Tablante, 2002; Dorea, 2010; Van Steenwinkel, 2011).

Vaccinating laying hens and breeder flocks against *Salmonella* is often practiced in the poultry industry. However, due to vaccine cost and issues with safety and vaccine effectiveness, vaccination of broiler flocks is often not performed. Instead, flocks are often only vaccinated after *Salmonella* infection has been confirmed (Desin, 2013; Gamazo, 2007).

After a flock contracts *Salmonella* there are several courses of action. The flock could be eradicated due to contamination and infection spread concerns, specifically if the *Salmonella* strain present is *S.* Gallinarum*, S.* Pullorum or *S.* Typhi. The affected flock could also be treated with antibiotics, however this practice is not always effective and the treated flock has to go through a mandatory withdrawal period prior to processing. In some cases, the infected flock will be designated as not fit for human consumption and rendered into pet food and other products. This is often the case when a flock fails the mandatory carcass rinse tests in the processing facility.

Methods of Surveillance

Salmonella is kept under surveillance by government agencies such as the Centers for Disease Control and Prevention (CDC), the United States Department of Agriculture (USDA), the Food Safety Inspection Service (USDA-FSIS) and Animal and Plant Health Inspection Service (USDA-APHIS) (Salmonella, 2015; Poultry Disease, 2015; Data Collection, 2015). Each agency addresses *Salmonella* surveillance differently and from different aspects but the overall main goal remains the same.

The CDC has several different programs that target *Salmonella* surveillance. Their programs target data collection and tracking for outbreaks associated with human disease and identifying the *Salmonella* serotype that caused that specific outbreak. The Laboratory-based Enteric Disease Surveillance (LEDS) program specializes in collecting data from clinical

diagnostic laboratories that confirm and identify *Salmonella* isolates from human samples. In addition, they publish an annual report based on their findings.

The National Notifiable Diseases Surveillance System (NNDSS) is an outbreak tracking system for all diseases that are reported as required by law. NNDSS collects data from local and state agencies that collected the initial report on diseases such as Salmonellosis and foodborne disease outbreaks. Like LEDS, they also publish their findings both weekly and annually.

The Foodborne Disease Active Surveillance Network (FoodNet) tracks foodborne illness trends as they relate to specific foods, environment, time and incidence of occurrence. Partnering with the USDA-FSIS, the Food and Drug Administration (FDA) and various state agencies, they provide a basis for disease prevention and food safety updates.

The National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) uses Pulsed Field Gel Electrophoresis (PFGE) to identify, track and link foodborne illness pathogens, which ultimately allows for the connection and identification of a foodborne illness outbreak. PulseNet is not managed exclusively by the CDC; local, state and national laboratories cooperate and collaborate to compare PFGE patterns, which ultimately allows for the identification and tracking of outbreaks.

The National Antimicrobial Resistance Monitoring System – enteric bacteria (NARMS) is similar to the CDC's other surveillance programs in the sense that it tracks foodborne illness disease outbreaks; however what makes them different is that they specifically target antibiotic resistance bacteria, including *Salmonella*. NARMS is also a partnership between the CDC, FDA, USDA and various health departments.

The Foodborne Disease Outbreak Surveillance System (FDOSS) is similar to FoodNet in that it collects and analyzes data that was reported voluntarily regarding foodborne illness

outbreaks and links disease occurrence to environment, specific foods and other factors. FDOSS is unique in that not only does it track outbreaks transmitted by food, it also tracks disease transmission by water, zoonotic transmission and direct person-to-person transmission (Salmonella, 2015).

The USDA-FSIS also has several programs that target *Salmonella* surveillance. Their programs target pathogen detection and reduction during various processing procedures. Currently there are five different inspection programs that poultry processing plants can opt into. The most recent addition implemented in 2014, The New Poultry Inspection System, is responsible for the postmortem inspection of meat animals as well as the microbial testing of carcasses and equipment in poultry processing plants. This new system targets pathogen reduction by requiring direct prevention of *Salmonella* occurrence through microbiological testing during production instead of control measures after the contamination occurs.

The Hazard Analysis and Critical Control Point (HACCP) system reduces the likelihood of contamination through very stringent process control measures based on of 7 basic principles: perform a hazard analysis, identifies control points, sets limits and monitoring requirements, determines corrective actions, recording and verification procedures for that specific process that greatly decreases the possibility of contamination. Participation in this program is mandatory under USDA-FSIS for poultry processing plants (Data Collection, 2015).

The USDA-APHIS is in charge of the National Poultry Improvement Plan (NPIP). The original purpose was to eradicate Pullorum Disease (*Salmonella* Pullorum) in poultry flocks. Today the program tests commercial and backyard flocks for several different types of *Salmonella* that cause disease in poultry and humans as well as *Mycoplasma* species and Avian Influenza (Poultry Disease, 2015).

From the descriptions of the various programs that target *Salmonella* surveillance, it is easy to see that there are many different surveillance methods that often overlap and target *Salmonella* occurrence during processing and after the product has reached the consumer. However, except for the NPIP there is little to no surveillance that directly addresses the prevalence of *Salmonella* pre-harvest unless an outbreak occurs. Additionally, practices associated with low incidences of Salmonella have not been identified nor has a baseline level of on farm *Salmonella* levels been established. The baseline could be used to test a flock and compare it against to determine if corrective procedures need to be made.

Current Regulations

The first regulation regarding meat products was the Meat Inspection Act of 1906 and was passed in response to the exposure of highly unsanitary processing conditions and the use of poisons during the meat processing and preservation process. All current regulations regarding biological contaminants stem from this initial Act in an effort to improve on the safety and wholesomeness of meat products (Significant Dates, 2014).

In 1957 the Poultry Products Inspection Act (PPIA) was enacted and gave the USDA-FSIS regulatory and inspection control over poultry processing facilities. Each facility was required to have an ante- and postmortem inspection of all birds going through the facility that would condemn any and all parts or carcasses that were deemed adultered and unwholesome. In addition, each facility was required to pass sanitation inspections per the direction of the USDA-FSIS (Regulatory Information, 2009). While this Act did not directly or specifically target *Salmonella*, it was the first step towards microbial testing of poultry products.

In 1996 the Pathogen Reduction/HACCP Rule was enacted under the regulatory control of the USDA-FSIS in order to reduce, control and prevent pathogens in raw food products.

Under this rule each facility must have a complete and approved HACCP plan that addressed all aspects of processing in that facility. This rule specifically targeted *Salmonella, Listeria* and *Escherichia coli* by setting performance standards and microbial testing procedures that would determine if the performance standards are being met (Key Facts, 2013). The *Salmonella* performance standards for broiler carcasses are a maximum of 7.5% positive samples, or 5 positive samples out of 51 total samples tested. Each processing facility is placed into one of four distinct categories based on the average test result. Each category is sampled at different time intervals depending on their performance, with those facilities that have a high incidence of *Salmonella* being sampled more frequently (Pathogen Reduction, 2015).

To further address the public health concern of *Salmonella* in meat products, the USDA-FSIS proposed the Salmonella Action Plan (SAP) that was then approved in 2013. The SAP focused on updating and improving the poultry slaughter and inspection processes by making food safety and pathogen reduction the main focus of inspectors in the processing plants. To attain these goals, the SAP used data collected from the Pathogen Reduction/HACCP Rule system to identify problem areas and the corrective actions needed to provide a solution. They specifically used the performance history of each processing facility and their *Salmonella* category during their analysis and implemented continuous rather than intermittent microbial testing. They also concluded that performance standards for raw poultry parts and ground poultry products should be set based on continued sampling and data collection. In addition, the SAP examined the pre-harvest factors that contribute to *Salmonella* contamination with the goal of identifying the practices associated with a low incidence of pre-harvest *Salmonella* contamination (Strategic Performance, 2013).

The SAP continued their *Salmonella* incidence reduction efforts by proposing new standards in 2015 for raw poultry parts and ground poultry products. The proposed changes is expected to decrease the number of illnesses caused by *Salmonella* by 30% (USDA Proposes, 2015). The *Salmonella* performance standard for broiler carcasses will remain the same; however ground chicken will have a maximum of 25% positive samples, or 13 positive out of 52 total samples. The performance standard for chicken parts will be 15.4% positive samples, or 8 positive out of 52 total samples (Changes to the Salmonella, 2015).

The ultimate goal for the USDA-FSIS is zero tolerance of *Salmonella* and other foodborne illness pathogens in poultry products. While this goal is admirable, the feasibility of executing this goal may prove harder than anticipated due to the ubiquitous nature of the organism and the current lack of pre-harvest prevention strategies.

Clostridium perfringens

History

Discovered in 1892 by William H. Welch and George Nuttall, *Clostridium perfringens* is a gram-positive, spore-forming, rod-shaped, obligate anaerobic bacterium. *C. perfringens* was originally known to cause gangrenous infection in both humans and animals but it wasn't associated with foodborne illnesses until L.S. McClung made that discovery in 1945 (Barceloux, 2012). This pathogen is non-motile, and like *Salmonella spp.*, is normally present in the environment and the natural gut micro flora of many animals.

The genus *Clostridium*, a member of the family *Clostridiacea*, has over 200 species and several subspecies, several of which are known to be pathogenic to humans (Public Health England, 2015). Of the pathogenic Clostridia, the most well known are *Clostridium difficile, C. botulinum, C. tetani* and *C. perfringens. C. difficile* is associated with prolonged or unnecessary

antibiotic use in healthcare facilities, especially in elderly or immunocompromised individuals (Healthcare-associated, 2015). *C. botulinum* is the infamous cause of botulism in humans. There are four types of botulism: infant, wound, foodborne and undetermined. In all four types, *C. botulinum*, its spores or endotoxins are the etiological agent (Bad Bug Book, 2014). *C. tetani* produces an exotoxin that when introduced into the body, usually via a wound, can cause tetanus in humans. A vaccine against this disease is widely used and available; however a booster vaccine is required every 10 years to protect against infection (Tetanus, 2015). *C. perfringens* causes several different diseases in humans, including food poisoning, necrotic enteritis (NE), gas gangrene, wound infections, and is thought to be a contributing factor in sudden infant death syndrome (SIDS) (Wells, 1996; Meer, 1997). In the United States, *C. perfringens* causes almost a million cases of foodborne illnesses each year making it the third top contributor to foodborne illnesses (CDC Estimates, 2014).

While it is not unusual for *C. perfringens* to be found in both human and animal intestines, illness in humans occurs upon the ingestion of large quantities of live bacteria, often from contaminated or improperly prepared food products. Food poisoning caused by this organism is associated with diarrhea and abdominal cramps. Symptoms generally persist for 24 hours or less and unlike other causes of food poisoning, vomiting and fever do not occur (Food Safety, 2014).

The symptoms of necrotic enteritis (NE) caused by *C. perfringens* typically include bloody diarrhea, abdominal distention, bowel perforations and hemorrhaging of the intestine (Wells, 1996; Severin, 1984). While this disease is rare in humans it is a common disease of domesticated poultry. In humans the disease is caused by ingestion of an infective dose of the live bacteria and is often associated with large meals that slow the pass through rate in the

intestine allowing the ingested bacteria to proliferate (Wells, 1996). In poultry, *C. perfringens* itself does not cause NE unless the intestine has been damaged by a secondary agent such as being parasitized by a coccidial species or being fed a diet that is difficult to fully digest such as one high in non-starch polysaccharides found in some cereal grains (Shojadoost, 2012).

Gas gangrene and wound infections occur when an open wound is exposed to material such as feces or soil that contain *C. perfringens*. Gas gangrene is a very serious illness with a high mortality rate that occurs when the wound area has been extensively damaged, reducing blood flow which creates an anaerobic environment that is ideal for this organism. Once the infection has been established, *C. perfringens* undergoes fermentation, which produces gases that pool in bubbles under the skin. At the same time it also produces exotoxins that break down the surrounding tissues and vascular system leading to extreme discoloration of the skin and muscle. Wound infections are generally less serious and more superficial in nature. They have a better prognosis than gas gangrene with a moderate treatment plan (Wells, 1996). *C. perfringens* also causes wound infections in poultry that is called gangrenous dermatitis. This disease has a high incidence of mortality and the exhibited symptoms are very similar to those expressed by gas gangrene in humans (Overview of Gangrenous, unknown).

It is important to note that while *C. perfringens* causes several different diseases in both humans and animals, often with symptoms that closely resemble each other, the strains that infect humans are generally different than those that infect animals. However, it is possible for animals, specifically poultry in this case, to silently carry a *C. perfringens* strain that is not pathogenic to them but highly infectious to humans (Epsilon Toxin, 2004).

The economic cost of *C. perfringens* is extremely high due to its ability to cause varied infections in both humans and animals and the number of cases that occur each year. According

to the USDA, the annual cost as of 2013 for human associated infections is an estimated $342.7 million (Cost Estimates, 2014). One study estimated that the yearly global cost of *C. perfringens* in poultry was $2 billion (Shojadoost, 2012). There are many factors that influence the combined economic cost of this disease including: treatment, prevention strategies, mortality, performance reduction and hospitalization.

Classification

C. perfringens strains are classified into five types, A-E, depending on the enterotoxins they produce, of which there are four types (Adair et al, 2008; Epsilon Toxin, 2004). Alpha toxins are produced by all five types of *C. perfringens* and causes hemolysis by misdirecting white blood cells away from the site of infection, reducing local blood flow due to vasoconstriction and altering cell metabolism. Beta toxin encoded by the cpb gene is expressed by *C. perfringens* Types B and C. The method of action for this toxin is not fully understood; however it has been suggested that it causes pores to form in affected cells that ultimately causes NE (Jihong, 2013; Shatursky, 2000). Epsilon toxin rarely infects humans but is extremely toxic to many animal species. Its method of action is to forms pores in affected cells and causes an increase in the permeability of the intestines which allows the toxin to enter the bloodstream and subsequently attack the nervous system causing an asymptomatic death in many cases. This type of toxin is very potent, second only to the toxins produced by *C. botulinum* and *C. tetani,* and is produced by *C. perfringens* Types B and D (Jihong, 2013; Wioland, 2013). Iota toxin is only produced by *C. perfringens* Type E and is made up of two different proteins that have different domains and purposes. One protein serves as the active portion able to produce a response while the other protein binds to receptors on the target cell (Jihong, 2013; Marvaud, 2002).

C. perfringens Type A produces several different types of alpha toxins as well as novel toxins such as *NetB*. Type A strains have been proven to be the major factor in gas gangrene in both humans and animals, as well as food poisoning in humans. Alpha toxin *CPE* has been implicated in Crohn's disease and food poisoning in humans while the novel toxin *NetB* has been implicated as the main cause of NE in chickens (Titball, 1999, Jihong, 2013). Type B strains produce both alpha, beta and epsilon toxins and have not been reported to infect humans and instead cause NE in livestock species (Jihong, 2013). Type C strains produce alpha and beta toxins that causes NE in humans, however because this type of infection is rare in humans, it is not considered a major public health issue. In many domesticated mammalian and avian species, this strain causes NE and enterotoxemia especially in young specimens. However, in chickens most cases of NE are caused by Type A and not Type C (Jihong, 2013; Shatursky, 2000). Type D strains produce alpha and epsilon toxins and are not known to cause disease in humans. Instead this strain causes enterotoxemia in domesticated livestock species but does not concurrently cause NE. Type E strains produce alpha and iota toxins, and like Type D, do not cause disease in humans. This strain causes enteritis in domesticated livestock species and rabbits (Jihong, 2013).

As already stated, *C. perfringens* causes disease in the host by producing toxins. Two genes, *NetB* and *TpeL* that code for two novel toxins have been suspected to be major virulence factors for the disease NE, especially in avian species. However, there has been little data composed on the subject concerning the prevalence of these genes in NE outbreaks and to determine how often they occur in *C. perfringens* isolates (Jihong, 2013; Bailey, 2015). Currently, the most popular method used to determine what toxins a *C. perfringens* isolate produces is through the Polymerase Chain Reaction (PCR). DNA primers specific for the toxin

gene sequence such as for *NetB* and *TpeL*, would indicate if that isolate possesses the genes necessary to produce these toxins (Songer and Meer, 1996).

Disease Transmission, Control and Treatment

Due to the ubiquitous nature of *C. perfringens*, it is not possible to completely eradicate this organism from the poultry production environment. Instead, efforts should be focused on preventing the diseases associated with this pathogen through various prevention strategies.

The disease NE occurs after the integrity of the gut wall and its environment has been compromised. As already stated, this can be caused by primary infection of the flock with coccidia or the use of difficult to digest feed ingredients such as wheat and other cereal grain that are high in non-starch polysaccharides and cause irritation and damage to the intestine allowing *C. perfringens* to colonize the affected area (Shojadoost, 2012).

Proper management techniques are necessary to control and reduce stress levels in flocks, which will reduce activity associated injuries through which *C. perfringens* can cause infection. Raising flocks in an environment previously associated with a NE outbreak that was not properly sanitized can lead to subsequent flocks being infected (Abd El-Ghany, 2010; Cowen, 1987). As with *Salmonella* prevention strategies, it is important to provide feed and water that are clean and uncontaminated to prevent disease transmission and promote a healthy immune system (Abd El-Ghany, 2010).

Flock vaccination programs, while effective in preventing many diseases can suppress the bird's immune system leading to an increased likelihood of infection and NE occurrence (McReynolds, 2004). Other common prevention strategies include the addition of anticoccidials or competitive exclusion products to the feed (Lanckriet, 2010; Tactacan, 2013). In the past, antibiotics have been used prophylactically to prevent *C. perfringens* infection in poultry flocks.

However, due to increased microbial resistance to antibiotics, the industry is moving away from using antibiotics as a preventative and instead is finding other ways to prevent microbial infections and only use antibiotics as a treatment for the disease if absolutely necessary (Tactacan, 2013).

For flocks diagnosed with NE or other diseases caused by *C. perfringens*, there are few treatment options due to the cost of antibiotics, their associated withdrawal period and the production reductions associated with the disease. In most cases, infected flocks are processed and the affected birds condemned.

Methods of Surveillance

C. perfringens is kept under surveillance through the CDC. While surveillance of this pathogen is not nearly as extensive as that for *Salmonella*, outbreaks of this pathogen and the illnesses it causes are still tracked and analyzed. The FDOSS and National Outbreak Reporting System (NORS) collect data on reported foodborne diseases outbreaks, including those illnesses caused by *C. perfringens*. Each year they produce an annual report that publishes their findings (CDC, 2015). Unlike for *Salmonella*, the USDA-FSIS does not keep *C. perfringens* under surveillance or directly try to control its occurrence on any level (Becker, 2010). However, the HACCP system and the in-plant ante- and postmortem inspection processes will indirectly reduce the occurrence of this pathogen by improving the overall quality, sanitation, and safety of the meat products and processing environment. Overall there is an astonishing lack of oversight in regards to this pathogen and its presence in poultry products, even though *C. perfringens* causes over a million cases of foodborne illnesses each year in the United States.

Current Regulations

The history of regulatory precedence for the control of *C. perfringens* was initially the same as it was for *Salmonella*. The PPIA of 1957 required ante- and postmortem inspections of all birds going through a processing facility and the condemnation of any parts or carcasses that were deemed adultered and unwholesome (Regulatory Information, 2009). As with *Salmonella*, this Act did not directly target *C. perfringens;* however, it was the initial step towards microbial testing of poultry products.

The Pathogen Reduction/HACCP Rule purposefully did not target *C. perfringens* in raw poultry products due to its ubiquitous nature and the probability that control measures for these bacteria at the processing stage would not be effective (Key Facts, 2013; Performance Standards, 1999). In 1999 the USDA-FSIS set performance standards for ready-to-eat poultry products. They mandated that the products must be stabilized during processing so that the finished product has no more than one Log_{10} of *C. perfringens* bacteria that proliferated during the cooling down period after a heat treatment was applied (Performance Standards, 1999).

While *C. perfringens* is not targeted nearly as harshly as *Salmonella* in raw poultry products, it is monitored in cooked, ready-to-eat products because of the ability of the organism to survive, thrive and produce spores under less than ideal conditions. Proper handling and thoroughly cooking raw poultry products will eliminate *C. perfringens* in the finished product. There are no pre-harvest preventative or monitoring strategies for this pathogen other than performing proper biosecurity and management practices. The ubiquitous nature of this organism would make it difficult to control in the pre-harvest environment, however at some point this may need to be addressed if complete pathogen elimination in poultry products is desired.

Campylobacter jejuni

History

Theodor Escherich is credited with the initial discovery of the genus *Campylobacter* in 1886. He found a spiral shaped organism in neonate and kitten fecal samples from those afflicted with diarrhea. Attempts to cultivate these organisms were not successful, as it would not grow on any solid media produced at the time (Kist, 1986). Originally called vibrio, this bacterium was first isolated by McFadyean and Stockman in 1906 during their investigation of epizootic abortion in livestock species. Unlike Escherich's discovery, diarrhea was not associated with this infection in livestock, which was ultimately found to be due to a different species of *Campylobacter*, known then as *Vibrio fetus* (Skirrow, 2006).

In 1931, Jones *et al.* isolated vibrios from the jejunum of cattle with diarrhea and then again from calves fed feces from the aforementioned cattle using a blood agar media that was able to support the growth of this bacterium. From their findings, Jones *et al.* proposed the name *Vibrio jejuni* that would later be changed to *Campylobacter jejuni,* as it is known today (Jones, 1931).

In the 1950s Elisabeth King found bacteria similar to those discovered by Jones *et al.* in human diarrhea samples. She investigated the differences between various vibrio isolates from blood samples and termed "related vibrios" in reference to *V. jejuni* and *V. coli* and noted that they were different than other vibrio isolates (Skirrow, 2006; Engberg, 2006).

Campylobacter was first officially isolated from human fecal samples by Butzler *et al*. in Belgium in the 1970s (Engberg, 2006). The formulation of selective media capable of sustaining *Campylobacter* allowed for the in depth studies of various isolates and the identification of multiple species of *Campylobacter*. Since then, *Campylobacter jejuni* has been identified as a human pathogen that is the leading cause of bacterial gastroenteritis in the United States. (Butzler, 2004).

The genus *Campylobacte*r is a member of the family *Campylobacteraceae* and currently has 30 species including *C. jejuni, C. coli* and *C. fetus*. As already stated, some of these species are associated with gastroenteritis or food poisoning in both humans and animals. Most species require anaerobic or microaerophilic conditions at a temperature between 25° to 42° C. Many species are motile and are present in the environment as well as the natural gut micro flora of many animals, including birds (Lastovica, 2014). Because of this, poultry are often a common source of infection for human consumers (Janssen, 2008).

Diarrhea, fever, abdominal cramping and pain that last for up to a week after initial infection characterize Campylobacteriosis, or the disease caused by *Campylobacter* spp. in humans. In some cases nausea and vomiting also occur while in others no symptoms are observable. While rare, infections outside of the intestines are possible including meningitis, bacteremia, septic arthritis, endocarditis and osteomyelitis. Anyone can get infected with *Campylobacter* upon the ingestion of an infective dose. However, those with a compromised immune system, the very young and young adults are especially vulnerable to infection (Campylobacter, General Information, 2014; Acheson, 2001). Many people contract this infection while traveling abroad but most people in the United States contract Campylobacteriosis from contaminated and undercooked poultry meat (Sheppard, 2009). *Campylobacter* infections have been linked to Guillain-Barre syndrome, an autoimmune disease that causes paralysis by affecting the nervous system. Bacterial infections, especially by *Campylobacter* are often preceded by the occurrence of this disease (Nachamkin, 1998).

There are an estimated 1.3 million cases of Campylobacteriosis every year in the United States. Many cases are never reported or diagnosed so the reported number of cases is vastly underestimated (Campylobacter, General Information, 2014). Occasionally multiple cases are

linked to each other and a *Campylobacter* outbreak identified. Recently in 2013-2014 an outbreak of *Campylobacter* was linked to the ingestion of improperly prepared chicken liver pate at restaurants that used poultry livers from the same poultry production facility in Vermont. There were six total cases, two of which were hospitalized but all fully recovered (Multistate Outbreak, 2013).

While *Campylobacter* causes diseases in humans, it rarely does so in avian species. In most cases the bacteria are naturally present in the gut micro flora and does not increase mortality or exhibit symptoms. However, several strains of *C. jejuni* have inconclusively been associated with the death and enteritis in chicks and poults (Overview of Avian, unknown).

The economic cost of *Campylobacter* infections is high due to its ability to cause foodborne illness in humans as well as potentially predispose them for the disease Guillain-Barre syndrome. According to the USDA, the annual cost as of 2013 for human associated infections is an estimated $1.9 billion (Cost Estimates, 2014). Since *Campylobacter* does not cause significant disease in poultry, there are no estimates available for the estimated cost of this infection in poultry.

Species of Importance

Of the 30 species in the genus *Campylobacter*, several are known to cause disease in humans. The specie that has the most significant impact on human disease is *C. jejuni*. One study found that between 58-78% of all isolates from human samples are caused by *C. jejuni* (Sheppard, 2009). The CDC also attributes the majority of foodborne illnesses caused by *Campylobacter* to this specific specie (Campylobacter, General Information, 2014). *C. jejuni* is also commonly associated with Guillain-Barre syndrome as a primary infection (Speed, 1987). There are two subspecies of *C. jejuni; C. jejuni* spp. *jejuni* and *C. jejuni* spp. *doylei*. Both

subspecies are capable of causing human disease, however, *C. jejuni* spp. *jejuni* is more commonly isolated from human samples. The major distinguishing feature between the two subspecies is that *C. jejuni* spp. *doylei* is not able to reduce nitrate, while *C. jejuni* spp. *jejuni* can. From a clinical perspective, both *C. jejuni spp*. are able to cause gastritis, however *C. jejuni* spp. *doylei* is also able to cause enteritis (Parker, 2007).

Second to *Campylobacter jejuni, C. coli* is most commonly associated with illness in humans, including Guillain-Barre syndrome (Speed, 1987). One study found that in 2000, *C. coli* caused over 25,00 cases of foodborne illness (Tam, 2003). Formerly called *C. fetus* spp. *jejuni*, this bacteria causes disease symptoms that include watery diarrhea as well as the typical Campylobacteriosis symptoms. *C. coli* infections are often transmitted by contaminated water as well as by food sources, with summer and fall being the peak time of infection (Campylobacter coli, 2011).

Campylobacter upsaliensis is not well known as a human pathogen, however recently it has been associated with spontaneous abortions, hemolytic-uremic syndrome and diarrhea (Bourke, 1998). In the past, this organism has been suspected as being transmitted to humans from dogs that are on a raw meat diet, however recent genetic analysis has shown that the strains that cause infection in humans are different from those carried by dogs. This indicates that dogs are not a reservoir of infection for humans (Damborg, 2008). In 1992 there was an outbreak of this organism in Brussels day care facilities that infected 44 children who presented with diarrhea as the primary symptom (Goossens, 1995).

Campylobacter lari formerly called *Campylobacter laridis* can cause bacteremia and gastroenteritis in humans and has been linked to prosthetic joint and pacemaker implants as a cause for post-operative infection. In most cases these infections were associated with

immunocompromised or elderly patients (Skirrow, 2006; Von Graevenita, 1990; Morris, 1998; Werno, 2002).

Campylobacter hyointestinalis infection causes watery diarrhea in humans although an asymptomatic infection can also occur (Skirrow, 2006). Like *C. lari* and *C. upsaliensis*, this pathogen is not well known as a human pathogen but nevertheless it is present and causes human infection.

Campylobacter concisus is unique among the *Campylobacter* species as it has been associated with oral diseases such as gingivitis and periodontitis as well as with various intestinal diseases. Recently this organism has been linked to those that have been diagnosed with Crohn's Disease and ulcerative colitis (Kaakoush, 2012). One study linked *C. concisus* to the causative agent of bacterial diarrhea in immunocompromised individuals (Aabenhus, 2002). Another study showed that individuals that have inflammatory bowel disease are infected by oral *C. concisus* strains (Ismail, 2012).

Campylobacter ureolyticus formerly known as *Bacteroides ureolyticus* has also been linked to those diagnosed with Crohn's Disease. The organism has also been isolated from skin infections, gangrenous lesions as well as being linked to those that also have oral diseases (Burgos-Portugal, 2012). Like other *Campylobacter* species, *C. ureolyticus* also causes gastroenteritis in humans (Bullman, 2011).

Disease Transmission, Control and Treatment

As already stated, poultry products are a common source of *Campylobacter* infection in humans. As such, controlling the presence of *Campylobacter* at both pre- and post-harvest points in the poultry production process is essential in preventing human disease. Pre-harvest contamination often occurs through a contaminated water supply or through the ingestion or

contact of contaminated fecal material. Vectors such as insects, wild birds and other animals, and farm personnel can also spread the bacteria (Campylobacter, General Information, 2014; Conlan, 2007). Vertical transmission rarely if ever occurs according to one study (Newell, 2003). Disease transmission often occurs when a *Campylobacter* colonized bird is processed because it does not show disease symptoms. When the contaminated viscera come in contact with chilling water or other carcasses, the bacteria is spread (Conlan, 2007). According to the CDC, the best way to prevent infection by *Campylobacter* is to thoroughly cook all poultry products, prevent cross-contamination by using separate utensils for raw and cooked products and repeated hand washing after handling of raw meat, pet excrement and touching those that have diarrhea (Campylobacter, General Information, 2014).

Similar to the control measures for both *Salmonella* and *Clostridium perfringens*, the most effective methods for controlling and reducing the transmission of *Campylobacter* in a poultry production environment is to have good biosecurity and management practices, an antibiotic or antimicrobial additive to poultry feed, competitive exclusion products and vaccination (Lin, 2009). However, due to increasing concern over general antibiotic resistance and *Campylobacter* strains that cannot be effectively controlled by antibiotics, this method of control and prevention is being phased out of use in poultry production. Instead, alternate measures are being explored.

Since *Campylobacter* species rarely cause disease in avian species, this type of infection is not usually diagnosed or treated in poultry.

Methods of Surveillance

Campylobacteriosis is kept under surveillance through the CDC. While surveillance of this organism is not as extensive as that for *Salmonella*, it is monitored more than *Clostridium*

perfringens. The CDC, in conjunction with other state and federal agencies, has several programs that it uses to keep *Campylobacter* under surveillance. The NNDSS tracks all diseases and outbreaks that are required to be reported by law by collecting data from 57 jurisdictions. The list of reportable diseases is updated annually and currently Campylobacteriosis is listed as a notifiable disease (National Notifiable, 2015). FoodNet is an important component of the CDC's Emerging Infections Program that collects data from various sites on an estimated 46 million people in the United States. They collect data on nine pathogens, including *Campylobacter,* that are commonly transmitted by food products (FoodNet Surveillance, 2015). PulseNet uses PFGE to identify, track and link foodborne illness pathogens and has been instrumental in identifying thousands of outbreaks including those associated with *Campylobacter* (PulseNet, 2013). NARMS is very similar to the CDC's other surveillance programs but what makes it unique is that this program targets foodborne disease outbreaks caused by antibiotic resistant bacteria including *Campylobacter* (National Antimicrobial, 2014).

Current Regulations

Like with *Salmonella* and *Clostridium perfringens*, the Meat Inspection Act of 1906 and the PPIA of 1957 indirectly positively impacted the occurrence of *Campylobacter* in poultry meat by making the finished product more wholesome (Significant Dates, 2014; Regulatory Information, 2009).

The Pathogen Reduction/HACCP Rule did not specifically target *Campylobacter* by setting performance standards or microbiological testing programs because they determined that by targeting *Salmonella* and *Escherichia coli* to reduce their numbers, will inadvertently lead to a reduction in the levels of other pathogens (Pathogen Reduction, 1998).

In 2011 the USDA-FSIS set performance standards for *Campylobacter* on whole carcasses of chickens. The *Campylobacter* performance standards for broiler carcasses was 15.6% positive, or 8 positive samples out of a 51 total samples tested (FSIS Notice, 2012). Recently, performance standards for ground chicken and chicken parts have also been established. The *Campylobacter* performance standard for ground chicken is 1.9% positive, or 1 positive sample out of 52 total samples tested. The performance standard for chicken parts is 7.7% positive, or 4 positive samples out of 52 total samples tested The goal of these performance standards for *Campylobacter* is to reduce the number of Campylobacteriosis cases by 33% (Changes, 2015).

While *Salmonella* has been a long-term target for pathogen reduction in poultry products, *Campylobacter* has only recently been targeted in the same manner. Because of this, there is less long-term information available that would help to control this pathogen at pre-harvest points instead of relying so heavily on regulatory standards and post-harvest sanitation measures.

Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction or PCR is often hailed as one of the most significant achievements and scientific advances in the last century due to its ability to exponentially magnify the genetic material of any organism even if only one strand of DNA is present in the original sample. Developed by Dr. Kary Mullis in 1983, this method has made it possible to study a wide range of subjects and target the entire genome or a single gene for analysis. The PCR has multiple applications including but not limited to use in: medicine, dentistry, forensics, pharmaceutical development, agriculture, clinical microbiology, and environmental science.

The PCR has several basic steps; first the sample solution containing the DNA molecules is combined with polymerases, nucleotides, and primers. The solution is then heated to 90-97° C

in order to denature and separate the DNA strands. Next, the temperature is reduced to 50-60° C so that the polymerases can synthesize duplicate strands of the original DNA. This occurs when the primers binds to each DNA strand which then allows the polymerases to attach nucleotides complimentary to the original strand of DNA. Lastly, the temperature is increased to 72°C so that the polymerases have optimal conditions to continue adding nucleotides and complete the DNA strand. Each cycle of PCR is composed of these three steps and results in double the DNA that was present at the beginning of the cycle. After approximately 20-30 cycles that take only a few hours to complete, millions of copies of the original DNA, or amplicons are present and sufficiently available for further study. After PCR is completed, the amplified solutions are then usually examined through gel electrophoresis to quantify the DNA present in the original sample and confirm the target DNA sequence was amplified (Johnson et al, 1991; Joshi and Deshpande, 2011; Hernandez-Rodriquez and Ramirez, 2012; Valones et al, 2009).

Three things have contributed to the widespread popularity and ease of use for the PCR method: specificity, sensitivity, and heat-stable polymerases. The specificity of this method comes from the primers used in PCR. There are many different kinds of primers available, and all select for specific DNA sequences allowing researchers to select only for the genes or areas of the genome that are of interest. Two primers are used for every PCR, a forward primer and a reverse primer. Both primers target the same gene but attach to a different DNA strand, either the reference strand or the complimentary strand. The sensitivity of this method comes from its ability to detect minute amounts of DNA through amplification that would be undetectable by most other methods. Original PCR protocols used polymerases isolated from *Escherichia coli*, however the temperature fluctuations that occurred during each cycle caused the polymerase to become inactive, rendering it useless for future cycles. This made the process both time

consuming and expensive as the polymerase had to be added at the beginning of each cycle. The discovery of *Thermophilus aquaticus* in hot springs and its polymerase now known as *Taq* solved this dilemma because it does not become inactive at high temperatures as this organism thrives in such conditions (Johnson et al, 1991; Joshi and Deshpande, 2011; Bartlett, 2003; Hernandez-Rodriquez and Ramirez, 2012).

Since its introduction in the 1980s, PCR has been used for thousands of applications, which required the original protocol for this method to be changed to attain the desired results. While the original protocol for PCR is relatively simple, recent introduction of advanced protocols and PCR equipment have led to several different variations of PCR depending on the subject matter, equipment used and goal of the method. In general there are two different types of PCR, qualitative and quantitative. Qualitative PCR selects and detects certain DNA segments and amplifies their numbers exponentially by making copies of the original DNA. More advanced methods are also able to quantify or measure the amount of the desired DNA segment that was in the original sample (Hernandez-Rodriguez and Ramirez, 2012; Joshi and Deshpande, 2011).

Multiplex PCR a qualitative PCR method that is able to select for and amplify multiple DNA sequences from the same sample, at the same time. Instead of using a single forward and reverse primer for one gene, forward and reverse primers for all target genes are used to select for the different DNA sequences (Hernandez-Rodriguez and Ramirez, 2012). In this study, multiplex PCR will be utilized to detect if the genes *NetB* and *TpeL*, which encode for novel toxins associated with NE in poultry are present in *C. perfringens* isolates collected from poultry litter samples.

III – Survey of Management and Biosecurity Practices on Broiler Farms from a Single Integrator in Alabama

ABSTRACT

Disease prevention on commercial poultry farms is an essential component to providing a safe, economical and sustainable meat product to consumers. Previously, the industry has addressed foodborne disease prevention through various post-harvest strategies, drug administration, genetics and biosecurity programs. However, biosecurity and management practices are the most recent target for preventative strategies. The goal of this study was to determine what influence the company integrator has on the biosecurity and management practices performed on individual poultry farms. A biosecurity and management survey was sent to all of the growers for a single integrator in Alabama. Based on the responses, strengths and weaknesses in the company's biosecurity program were identified, and recommendations for improvement made to company management. A second biosecurity survey was then sent to determine if any changes in biosecurity practices could be noted. The response rate was 27.50% for the first survey, and 24.80% for the second survey. There were no statistically significant differences between the two surveys for any biosecurity or management practice. Conversations with several growers indicated that the company made no effort to change their biosecurity program, which explains the lack of statistical significance. However, there were observable differences between surveys for several biosecurity practices. There was a 10% increase in having a rodent control program (100%), an 18.77% increase in having an insect control program (96.77%), and a 7.88% increase in shoe cover use (35.48%). There was also a 6.15% decrease in having a house entry and exit log (3.23%), 6.73% increase in workers having contact with poultry not on the farm (16.13%) and a 6.45% decrease in footbath use (93.55%). Findings from

this study were inconsistent with those from similar biosecurity surveys performed in the industry and are most likely due to different target demographics, local conditions and method of survey delivery.

INTRODUCTION

The goal of the poultry industry is to provide an economical, safe and sustainable meat source for consumers while maintaining a profitable market. With this goal, disease prevention on the commercial poultry farm is an essential component to providing such a product. Each year the US poultry industry, worth $48.3 billion annually, loses an estimated 10-20% of the industry's total value due to disease and its associated costs of product condemnation, recall, productivity losses, disease treatment, and legal action (Poultry, 2015; Biggs, 1982; Poultry and Animal Health, 2015). In the past, the industry has combated disease through breeding programs, drug administration, biosecurity programs, and post-harvest strategies (Biggs, 1982 Dorea et al., 2010; Bauermeister et al, 2008). Changing legislation and consumer acceptance on the allowable levels of certain pathogens such as Salmonella in poultry products, and the use of antibiotics in animals, has encouraged the industry to examine pre-harvest disease prevention strategies more closely (Changes, 2015; Singer, 2006). While having good biosecurity practices has long been associated with the prevention of disease, the recent emergence of Avian Influenza and the zoonotic risk it poses has also served to emphasize the importance of these practices.

Biosecurity is defined as "the protection of agricultural animals from any type of infectious agent…" and in the poultry industry include practices such as footbath use, disposable shoe covers worn inside houses and restricting farm access to necessary personnel (Biosecurity, 2012). In the United States, there is not a universal biosecurity program applicable to each individual poultry production unit, instead there are many factors such as flock status, location, equipment and housing available, that must be taken into consideration when implementing a biosecurity program (Butcher and Yegani, 2008). Because of the lack of a universal program and industry oversight, there are companies and farms that have substandard biosecurity practices,

creating a “weak link” in the overall system that could allow for disease to flourish. Additionally, while each poultry company has its own biosecurity program, the grower is ultimately responsible for the cost and day-to-day implementation of these practices. Based on the collective knowledge and interest of the company, and the one directional flow of information regarding biosecurity practices, it is evident that it is the company that is responsible for providing a list of expected biosecurity practices and educational materials to each grower and conveying the importance of these practices. However, according to one study, they found that many companies did not have a set of written guidelines and educational materials or only occasionally confirmed their use (Dorea et al., 2010).

To date, there have been many biosecurity surveys performed in the poultry industry (Dorea et al., 2010; Siekkinen, 2012; Ali, 2014; East, 2007). However, none have examined the influence that the company has on the actual biosecurity practices performed on the individual farm. The purpose of this study is to collect data via a survey on all of the management and biosecurity practices performed on the farms for a single integrator with the intent of identifying strengths and weaknesses in their biosecurity program. General recommendations will be made to the company based on the results, and the growers resurveyed to determine if any changes in biosecurity practices are evident.

MATERIALS AND METHODS

Experimental Design

In September of 2013 a survey was sent by mail to all of the broiler growers for a single integrator in Alabama (n=120). Based on the survey results, suggestions were made to the company’s management regarding upgrading certain biosecurity procedures. In September of 2014, the same survey was sent to all of the growers for the same integrator (n=125). Data from

both survey sets was entered into an Excel spreadsheet for comparison and then statistically analyzed.

The questionnaire that was developed for use in this study was based on biosecurity and management practices commonly performed and recommended by the poultry industry. (Pierson, unknown; Biosecurity, 2007; Dorea et al., 2010). Biosecurity questions characterized the most common practices performed on the farm, such as having a rodent or insect control program, using footbaths, restricted farm access and shoe cover use. Management questions also characterized the most common practices performed on the farm and focused on litter management, house management and common procedures performed between flocks. Additional questions were about general farm characteristics, such as size of the farm, number of birds per flock, mortality percentage, grow-out period length, and type and dimensions of poultry houses. A full summary of the questions contained in the survey is listed in Table 3.1.

All survey information and communications with the growers was kept anonymous so that none of the submitted information could be linked to a specific grower. All surveys were sent directly to the grower by mail and returned in the same manner. While company management did approve the contents of the survey, they were not involved in survey distribution or data collection. Anonymity was preserved to encourage accurate responses from the growers.

Data Analysis

Data from the survey was entered into an Excel spreadsheet. For each survey set, the percent response rate was calculated for each question, along with the minimum, maximum and average. Using SPSS (version 22.0.0.0), a comparative analysis was performed for all questions

over the two survey sets using either the chi-squared or t-test as was relevant to the individual questions using $\alpha = 0.05$ and p-value ≤ 0.05 was considered statistically significant.

RESULTS

Survey Responses

Of the 120 surveys that were initially sent to the broiler growers of a single integrator, 32 surveys (27.50% response rate) were filled out and returned. Responses to this survey were anonymous and were collected over a period of 45 days. For the second set of surveys, of the 125 surveys sent to broiler growers, a total of 31 surveys (24.80% response rate) were filled out and returned. 11 surveys were returned to us by the postal service as undeliverable making the total number of surveys that reached the growers for this set of surveys 114. Responses to this survey were also anonymous and were collected over a period of 47 days. While there were many responses to both sets of surveys, in some instances, especially for the open-ended questions, the responses were unreadable or not applicable to the question of interest. In these cases, the individual responses were excluded and not taken into consideration when calculating the percent response rate.

Company Recommendations

Based on the results from the first survey, it was determined that less than 75% of the surveyed farms treated the litter or cleaned the houses between flocks. In addition, basic biosecurity practices had inconsistent compliance, such as having a rodent control program (90%), insect control program (78%), shoe cover use (28%), and restricted farm visitors to necessary personnel (78%). Other practices had less than 10% compliance, such as farm entry and exit log, house entry log, shower in/out, or had visitors change their clothing before entry to the houses. Also, three surveys (9.4%) indicated that they had contact with poultry other than the

birds on the farm. The only biosecurity practice that had 100% compliance was footbath use on the farm. From these results, the general recommendation was to enforce the implementation of the biosecurity practices found lacking according to the results from the initial survey.

Farm Characteristics

Table 3.2. exhibits the average, minimum and maximum values for all of the farm characteristics questions on the broiler grower survey. The average grow-out period for survey one was 36.5 days, and for 35.88 days for survey two, and was statistically significant, t (61) = 2.147, p = 0.036. The average number of houses for both surveys was within 0.2 houses of each other, with survey one being 3.2 and survey two, 3.4 houses. The average number of houses between the two surveys was not statistically significant t (61) = -0.582, p = 0.563. The average and minimum house dimensions for both surveys were the same. The average house dimension was 40 ft by 400 ft and the minimum house size was 40 ft by 200 ft. The maximum reported dimensions for the second survey were much larger than those for the first survey. There was no statistical significance for house dimensions between the two surveys, $\mathbf{C^2}$ (7, N = 206), p = 0.933. There was a 100% response rate for the grow-out period, number of houses, and house dimensions questions.

The average house age for survey one was 18.28 years, approximately 1.5 years younger than the average house age for the second survey and was not statistically significant, t (203) = -1.732, p = 0.159. The maximum house age between the surveys had a difference of almost twenty years with the first survey having the oldest reported house. The average, minimum and maximum number of birds on each farm was different for both survey sets but was not statistically significant, t (60) = -0.986, p = 0.298. The average numbers of birds on each farm for survey two had approximately 12,000 more birds than set one. Survey two also had

approximately twice the number of birds reported for both the minimum and maximum categories. For both the age of houses and number of birds' questions, the response rate was 97% for survey one and 100% for survey two. The average number of flocks per year was 7 for survey one, and 6.52 for survey two and was not statistically significant, t (59) = 1.829, p =0.073. The maximum number of flocks for both surveys was the same at 7 flocks per year. For the number of flocks' question, the response rate for survey one was 100% but 96.77% for survey two.

The average percent mortality for the first survey was 1.57%, and 2.2% for the second and there was no statistical difference between the two surveys t (46) = -1.218, p = 0.229. The minimum reported mortality was 0% for both surveys and the maximum reported mortality was 5% for the first survey set and 7% for the second. The response rate for the percent mortality was 78% for set one and 77.42% for set two. Lastly, for the first survey the majority of the poultry houses were conventional style (62) and the remainder was tunnel style (40). For the second survey, the opposite is true, with the majority of the houses being tunnel style (53) and most of the remainder conventional (50), 3 "other" styles of housing were reported for this survey. The housing types between the two surveys was statistically significant, $\mathbf{C}^2$ (2, N = 208) = 6.03, p = 0.049. There was a 100% response rate for this question on both surveys.

Management Practices

Table 3 shows a summary of the results for several of the management related questions. The majority of farms reported that they use metal brood fencing, followed by plastic, both or "other", however, there were no statistically significant differences between the two surveys, $\mathbf{C}^2$ (3, N = 48) = 1.946, p = 0.584. This question had a 78.13% response rate for survey one and a 74.19% response rate for survey two. The majority of growers indicated that they use pine

shavings as the bedding source while the remainder used pine sawdust or a combination of the two bedding types. The first survey had a 97% response rate to this question and the second survey had a 100% response rate and there was no statistical significance between the two surveys, $\mathbf{C^2}$ (2, N = 62) = 0.693, p = 0.707. Used litter is removed from the poultry houses approximately once per year as indicated by both surveys. Some responses indicated they never changed the litter while others indicated they changed their litter after every flock. The response rate for this question was 94% for survey one and 90.32% for survey two. The survey results indicated that most growers disposed of their used litter by using it as a fertilizer, while others sold the used litter. Several respondents indicated that they both sold the used litter and used it as a fertilizer. There was a 94% response rate for this question on survey one and a 96.77% response rate on survey two, and there was no statistical significance between the two surveys, $\mathbf{C^2}$ (2, N = 60) = 1.562, p = 0.458.

The last management question on the broiler grower survey was an open-ended question that asked the respondent to describe what house management and preparation procedures are commonly performed between flocks. From the received responses, the answers to this question could be broken down into three categories: litter management, house management, and house set-up practices. There was a 91% response rate overall for this question on the first survey and a 96.77% response rate for the second survey.

Figure 1 illustrates the results for the litter management practices portion of the aforementioned question. There was a 75% response rate for the first survey and an 83.87% response rate for the second survey. From the graph we can see that over the two surveys, approximately 65-70% of respondents indicated that they de-caked the litter between flocks. This was the most common litter management practice performed and was followed by new shavings

added, insecticide, windrowed and litter tilled in decreasing order of occurrence. The two practices with the least amount of compliance was application of an acidifying treatment, with 5 responses for survey one and 4 responses for survey two. The practice that had the fewest responses was litter dried with one response each for both surveys. In all cases except litter tilled, the second survey had the same number of responses or less in each category when compared to the first survey. There was no statistical significance for any litter management category between the two surveys, $\mathbf{C}^2$ (6, N = 131) = 2.657, p = 0.851. Based on the responses for the litter management practice portion of the management question, it was apparent that many surveys performed multiple litter management practices as shown in Figure 3.2. From this figure it is evident that the majority of respondents performed three of the seven indicated litter management practices while five respondents for both surveys performed four or more practices. Conversely, ten respondents from survey one and fourteen respondents from survey two indicated they performed less than three litter management practices. There was no statistical significance between the two surveys for the number of litter management practices performed, t (48) = 0.759, p = 0.452.

Figure 3.3 shows the results for the house management practices portion of the last management question on the survey. There was a 62.5% response rate for the first survey and a 70.97% response rate for the second survey. From this graph it is evident that the category with the highest incidence of compliance was clean equipment as 60-68% of respondents indicated they performed this practice. The second most commonly performed practice was blowing down the houses with an air hose, followed by repairs, close houses and heat in descending order of occurrence. Heating the houses had one response each for both surveys. Unlike for the litter management practices, in every category, survey two had the same or more responses when

compared to survey one. There was no statistical significance between the two surveys for any house management category, $\mathbf{C}^2$ (4, N = 63) = 0.192, p = 0.996. Figure 3.4 shows the number of respondents that indicated they performed more than one house management procedure. For survey one, half of the surveys indicated they performed only one practice while the other half indicated they performed more than two practices. For survey two, just under half of the respondents indicated they performed two of the practices while only 18% indicated they performed more than two practices. There was no statistical significance for the number of house management practices performed between the two surveys, t (38) = -1.024, p = 0.312.

Figure 3.5 exhibits the results for the house set-up practices portion of the last management question on the survey. This portion of the question had the lowest response rate at 28.1% for survey one and 6.45% for survey two. From Figure 3.5, it is evident that survey one had almost the same number of responses for all four categories. Equipment set-up, new feed placed and house set-up procedures performed all had three responses each while brooder set-up had two responses. For survey two, the only categories that had responses were equipment set-up and house set-up with one response for each category. As with the previous portions of this question, Figure 3.6 shows the number of respondents that gave multiple responses. Eight of the nine responses for this portion of the question for survey one only performed one practice, while one response indicated they performed three of the four practice. Both respondents for survey two only performed one practice.

Biosecurity Practices

Figure 3.7 shows the results from the multiple choice, biosecurity practices question. There was a 100% response rate for this question on both surveys. Overall, compliance with the basic biosecurity practices such as having a rodent control program, insect control program,

visitors limited, footbath use, and no contact with other poultry, was high for both surveys. Compliance with these practices was between 65-100% for both survey sets. All other biosecurity practices except coveralls and shoe covers, such as farm/house entry logs, shower in/out, hand sanitation in houses and clothing change before entering houses had poor compliance with fewer than 5 responses (16% or less) for each category. The last option in this question, "other" allowed the respondent to fill in any additional biosecurity practices performed on their farm. For survey one, 4 responses were recorded for this category with responses ranging from excluding wild animals from the farm, biosecurity signage and gate use to exclude nonessential visitors. All of these responses could be categorized into one of the categories already on the survey. For survey two, there were two responses to the "other" category, both responses were about shoe covers and either indicated they were only used by visitors or only used when the area was under quarantine. Between 28-35% of the surveys for both sets indicated that coveralls and shoe covers were used, however in most cases the respondents indicated that only shoe covers were actually used. There was no statistical significance for any biosecurity practices category between the two surveys, $\mathbf{C}^2$ (11, N = 366) = 3.688, p = 0.978.

Figure 3.8 shows that many surveys indicated they performed more than one biosecurity practice. For survey one, the highest number of individual biosecurity practices performed on the farm was 10 with the minimum number being three practices. The majority of the respondents indicated they did between five and seven practices and accounted for 65% of the total responses. For survey two, the highest number of individual biosecurity practices performed on the farm was nine, with the minimum number being three practices. The majority of respondents indicated they did either five or six practices with nine counts each or 58% of the total responses

for that survey. There was no statistical significance between the two surveys for the number of biosecurity practices performed, t (61) = 0.084, p = 0.933.

DISCUSSION

Biosecurity and management practices are essential tools used to prevent disease in the poultry production system. Each company has their own biosecurity program and is responsible for introducing the importance of these practices to each individual grower as well as confirming that the company standards are being met. As the primary knowledge source for growers, it is imperative that each company implements a biosecurity program that will be effective at preventing disease while following industry recommendations. In the event that a biosecurity program is found lacking, it is essential that the company have the flexibility to adjust its program and enforce implemented changes when necessary. As the results from this study indicate, the willingness or ability to change the current biosecurity program is not always a priority. Overall, there was very little statistical significance between the two surveys. Only two farm characteristics questions showed any significance, and are most likely due to different growers responding to the second survey. The management and biosecurity questions did not exhibit any statistical significance for either of the two surveys. Based on conversations with several of the company's growers, they indicated that the company did not mention any of the results from this study nor suggest any changes to their current biosecurity program. This confirms that no effort was made to change the current biosecurity program and as such resulted in no significant changes. This is probably because there is not a direct or immediate benefit as perceived by the company that would cover the cost incurred by implementing changes.

While there were no statistically significant changes in biosecurity practices, there were some observable differences in the second survey when compared to the first survey. There was

100% compliance with having a rodent control program, which was an increase of 10% over the first survey. Also notable was an 18.77% increase in having an insect control program (96.77%), and a 7.88% increase in shoe cover use (35.48%). The observed increase in rodent and insect control probably is due to a previously inconsistent or nonexistent program that allowed for these pests to become established on the farm resulting in a need for extermination. The number of respondents that indicated they restricted farm visitors, had a house entry/exit log, shower in/out, visitors changed clothing before entering facility, restricted domestic animal access to houses, and washed hands in between houses remained the same with no change in the number of responses between the two surveys. Several practices showed a decrease in compliance on the second survey; house entry and exit log use decreased by 6.15% (3.23%), contact with poultry outside the farm had a 6.73% increase (16.13%), and footbath use, which previously had 100% compliance, decreased 6.45%. The decrease in having a house entry and exit log and footbath use could be due to new growers that initially followed protocol but ceased to do so after becoming established within the company. The increased contact with poultry outside the farm is likely due to growers assisting their acquaintances and family with their farms or due to backyard poultry flocks kept for youth programs such as FFA or 4-H.

The results also indicate that while the growers are somewhat consistently following the same biosecurity and management practices, many common biosecurity practices have poor compliance. In addition, compliance on paper does not necessarily mean the biosecurity practice is effective. Our team visited several of the grower's farms for a separate study during the time period that the surveys were being collected and noticed that while biosecurity measures were in place, they were not always properly maintained or performed. For instance, while all of the farms visited had footbaths, some were left outside, and were diluted by rain or heavily

contaminated with organic matter. In addition it was evident that the disinfectant solution is rarely changed. In all of these cases, the footbath solution is not optimal and would result in little to no disinfectant activity.

Perhaps one of the most important factors that effects biosecurity compliance other than education is cost. Growers are responsible for all costs related to biosecurity practice implementation. According to one study in Finland, the comprehensive cost of biosecurity during a disease free period for each bird is 3.55 eurocents ($0.04 USD) per bird resulting in a total of approximately $3053 per 75,000 birds (Siekkinen, 2012). On average, in 2011 U.S. growers received 5.55 cents per live-weight pound, and make about $25,000 per house per year (MacDonald, 2014;Vertical Integration, 2012). When farm and poultry house loan repayment and other expenses are taken into consideration, it is clear that biosecurity costs take up a significant portion of the grower's income. Therefore, if the grower does not understand the importance of biosecurity practices, they may be less likely to maintain them in order to keep costs low. In addition, one study found that biosecurity practice compliance was low during disease free periods but increased when a disease outbreak was present (Dorea et al., 2010). This suggests that while the growers may not see the benefit of having biosecurity practices in place in the absence of disease, they do recognize the need and benefit when an outbreak occurs. This is counterintuitive as biosecurity practices are meant to prevent the spread of disease and are equally important during times of health as a weak link in the system could lead to disease transmission and ultimately an outbreak. Once an outbreak occurs it is often too late for many flocks, as the disease has already spread.

When compared to past biosecurity surveys performed in the poultry industry, the average farm characteristics from this survey were similar. Our response rate of 24.8-27.5% was

similar to the response rate of 21.6-28.8% for Dorea et al. (2010), but considerably lower than the 38% response rate received by Tablante et al. (2002), and 49-70% response rate received by Vieira et al. (2009). The difference in response rate is most likely due to the target demographic and method of survey delivery being different than the previous studies. The average number of houses for our survey was 3.3 houses and was consistent with the number of houses found by Dorea et al. (2010) and Vieira et al. (2009) at four houses per farm, and 2.5 houses for Tablante et al. (2002). The maximum number of birds per farm as indicated by our survey was 77,000 and was slightly less than the 82,000 bird per farm population found by Vieira et al. (2009), and 96,000 total birds per farm by Tablante et al. (2002). The difference in the number of birds per farm is likely due to the target live weight being different than for previous studies or different average house dimensions. Lastly, the percentage of respondents that answered our farm mortality question was 78% and was very similar to the 76% that responded to Vieira et al. (2009).

The management practices commonly performed on broiler grower farms were inconsistent with previous findings. According to Dorea et al. (2010), only 10% of their respondents indicated they kept more than six flocks on the same litter, and 45% kept two to six flocks on the same litter, while our survey indicated that approximately 58% over the two surveys kept 6-7 flocks on the same litter, only changing it approximately once per year. This decreased number of litter change with this integrator can be attributed to the growers trying to maximize their profits, since the cost of new bedding can be substantial. Our survey also indicated that we had a much higher incidence of growers that decaked their litter (80%) compared to 62.5% found by Tablante et al. (2002), and 46% for Vieira et al. (2009). The increase in decaking litter can be attributed to the litter being used longer, since decaking will

help prolong the lifespan of the litter. Lastly, our survey indicated 68% of growers disposed of their litter by using it as a fertilizer and 23.4% sold the used litter. Vieira et al. (2009) found that 20% disposed of used litter by using it as a fertilizer, and 79% sold the litter for off farm use. The differences between the studies can be attributed to the location of the farms and the relative land availability to use the used litter as fertilizer.

Similar to management practices, the biosecurity practices commonly performed on the surveyed farms was highly inconsistent with the findings from other biosecurity surveys in the poultry industry. Our respondents had a higher incidence of compliance for several questions including footbath use, contact with poultry outside the farm, and domestic animals coming in contact with the birds on the farm. Our respondents had 96.5% overall compliance with footbath use, compared to 29.1% found by Dorea et al. (2010). Approximately 14% of respondents had contact with poultry outside the farm compared to the much higher findings of 35.2% for Dorea et al. (2010) and 40% for Vieira et al. (2009). Only 33% of respondents indicated they allowed domestic animals access to the poultry houses, compared to the 91.3% found by Tablante et al. (2002). The differences in these biosecurity practices can most likely be attributed to the standards maintained by the integrator being different than the standards for the integrators surveyed by previous studies. Conversely, our respondents also indicated they had decreased compliance with the following biosecurity practices when compared to other surveys: shower and clothing changed before entering or exiting farm, shoe cover use in the poultry houses, and hands washed when visiting different poultry houses. Dorea et al. (2010) found that 45% of their respondents showered and changed clothing before entering or exiting the poultry farm. Our surveys indicated that only 3% (one respondent) showered and 0% changed clothing. Dorea et al. (2010) also found that 83.4% of their respondents used shoe covers and more than 80% washed

hands between houses. Our survey indicated that approximately 32% used shoe covers and only 15% washed hands between houses. When compared to previous studies, the decrease in these specific biosecurity programs could be due to the purpose of the flocks raised by the growers. Broiler breeder and laying hen flocks often have stricter standards than those for broiler flocks.

While the results of our survey were somewhat inconsistent with the findings from other surveys, this is probably in part due to the different target demographics, local conditions and method of survey delivery. For instance, Vieira et al. (2009) surveyed multiple integrators at several different locations in Georgia and collected data on all poultry farms in those areas, including broiler growers and breeder-layer farms. Tablante et al. (2002) surveyed three integrators in the Delmarva Peninsula and Dorea et al. (2010) surveyed 8 integrators in North and South Georgia. Both Vieira et al. (2009) and Dorea et al. (2010) visited company management and service personnel in person and conveyed the goals of the project as well as an in depth discussion on the survey contents. Tablante et al. (2002) mailed the survey directly to the growers. In addition, the growers in northern Georgia were experiencing an outbreak of Infectious Laryngotracheitis (ILT) during the period of time the survey by Dorea et al. (2010) was distributed. The target demographic for this study was a single integrator in Northern Alabama that was not experiencing an outbreak of any sort and the survey was mailed directly to the growers without involvement of service personnel. Another factor that should be taken into consideration when comparing different biosecurity surveys, is the wording used for each question. Due to the different authors and target audiences the wordage used is different for each survey and as such may influence the responses received.

Future planned research in this area will be directed towards linking which specific factors or biosecurity practices directly impact the spread of disease. Farm sampling and

microbial analysis will be performed in conjunction with a survey of current biosecurity practices.

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Table 3.1. Summary of the survey questions that was sent to all of the growers for a single integrator in Alabama for the purpose of measuring the biosecurity, management and farm characteristics.

Question	Question Type - selections available	
Farm Characteristics		
Date survey was filled out	Open-ended	
Date received chicks for current flock	Open-ended	
Length of grow-out	Open-ended	
Number of houses on the farm	Open-ended	
House Dimensions	Open-ended	
Age of house(s)	Open-ended	
Number of birds on the farm	Open-ended	
Number of flocks per year	Open-ended	
Percent mortality in current flock	Open-ended	
Type of housing	Multiple choice	Circle one: conventional, tunnel, other.
Type of brood fencing used	Open-ended	
Management Characteristics		
Description of house management and preparation procedures performed between flocks	Open-ended	
Type of bedding material	Open-ended	
How often litter is removed	Open-ended	
How is litter disposed of	Open-ended	
Biosecurity Practices		
What biosecurity measures are performed on the farm	Multiple choice	Circle all that apply: rodent control program, insect control program, farm visitors limited, farm entry/exit log, house entry log, foot baths, soap and water/sanitizer available in each house, shower in/out, visitor change clothing before entry, visitors put on coveralls and shoe covers upon arrival, other animals restricted, no other poultry on farm or workers having poultry, other

Table 3.2. Farm characteristics questions and results for both surveys. Results displayed by number of respondents (Percent responded).

	Response rate (percentage)		
	Survey # 1	Survey # 2	P-value
Grow-out period	32 (100.00)	31 (100.00)	**0.036**
Average (days)	36.5	35.88	
Minimum (days)	35	34	
Maximum (days)	40	39	
Number of houses	32 (100.00)	31 (100.00)	0.563
Average	3.2	3.4	
Minimum	1	1	
Maximum	9	8	
House dimensions	32 (100.00)	31 (100.00)	0.153
Average (WxL)	40x400	40x400	
Minimum (WxL)	40x200	40x200	
Maximum (WxL)	42x500	52x640	
Age of houses	31 (97.00)	31 (100.00)	0.085
Average (years)	18.28	19.74	
Minimum (years)	1	2	
Maximum (years)	56	38.5	
Number of birds on farm	31 (97.00)	31 (100.00)	0.328
Average	71,935	83,774	
Minimum	12,000	25,000	
Maximum	165,000	300,000	
Number of flocks per year	32 (100.00)	30 (96.77)	0.073
Average	7	6.52	
Minimum	6	4.5	
Maximum	7	7	
Percent mortality	25 (78.00)	25 (77.42)	0.229
Average	1.57	2.2	
Minimum	0	0	
Maximum	5	7	
Type of housing	32 (100.00)	31 (100.00)	**0.049**
Conventional	62	50	
Tunnel	40	53	
Other	0	3	

Table 3.3. Management characteristics questions and results for both surveys. Results displayed by number of respondents (Percent responded).

	Response rate (percentage)		
	Survey # 1	Survey # 2	P-Value
Type of brood fencing	25 (78.13)	23 (74.19)	0.584
Plastic	8	5	
Metal	12	15	
Both	3	1	
Other	2	2	
Type of bedding material	31 (97.00)	31 (100.00)	0.707
Shavings	22	19	
Sawdust	8	11	
Both	1	1	
How often is litter removed	30 (94.00)	28 (90.32)	N/A
Average (times/year)	1.06	0.99	
Minimum (times/year)	0	0	
Maximum (times/year)	7	4	
Litter disposal method	30 (94.00)	30 (96.77)	0.458
Sold	9	5	
Used as fertilizer	19	22	
Both	2	3	

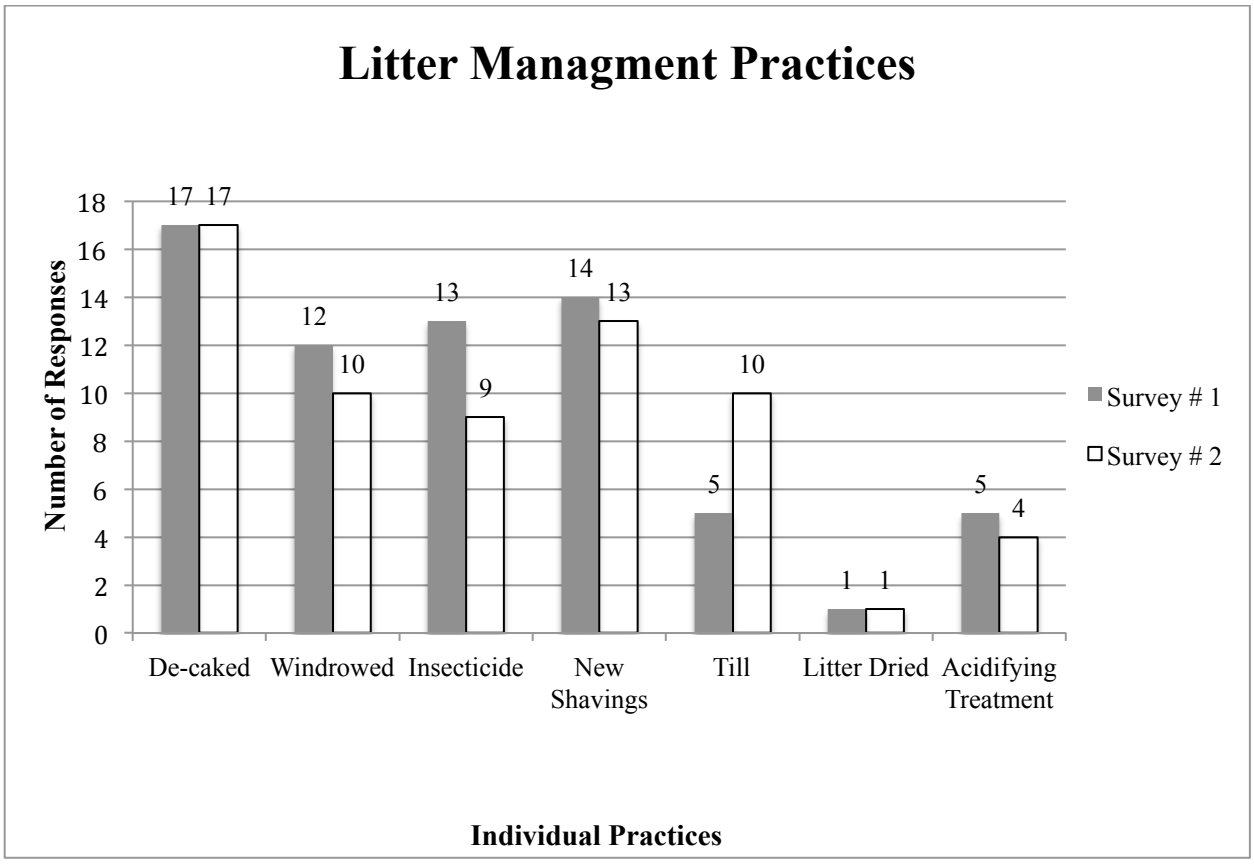

Figure 3.1. Number of responses for individual litter management practices for both surveys.

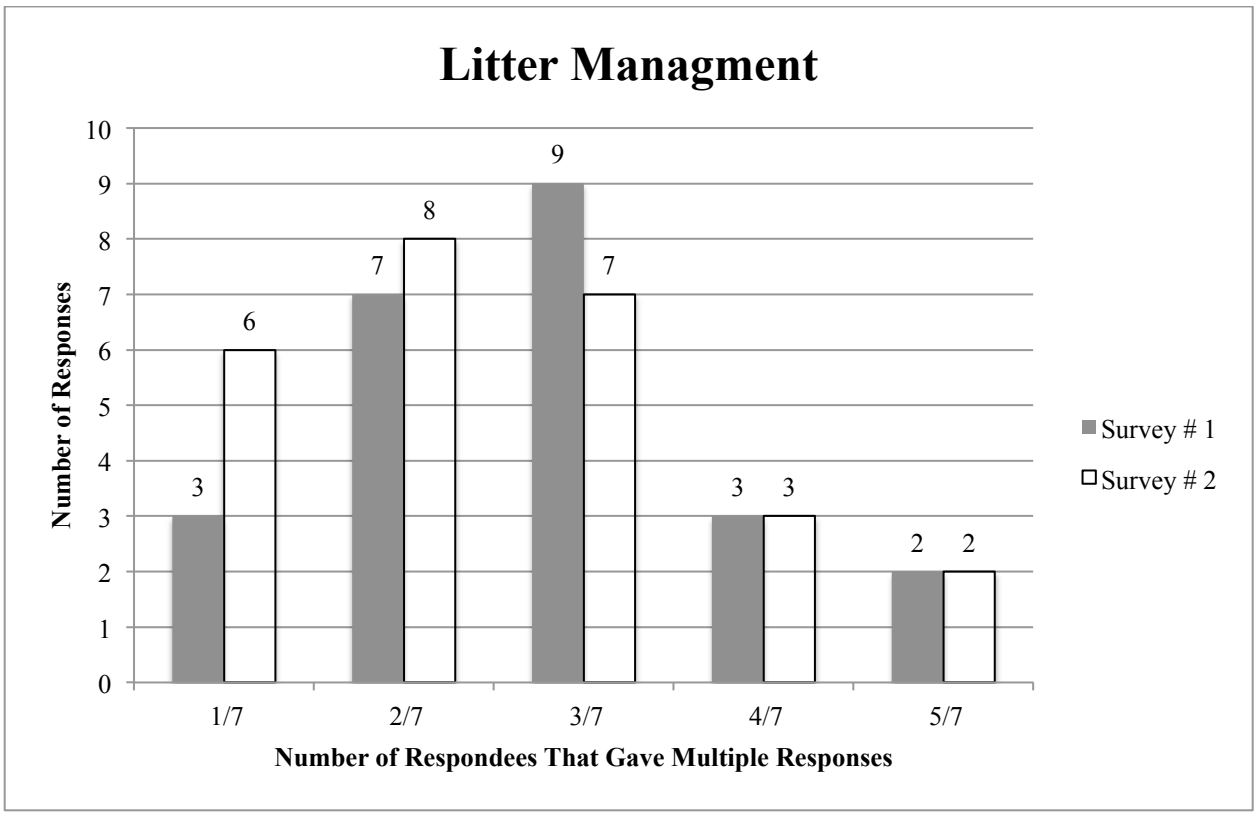

Figure 3.2. Number of responses for litter management that indicated they performed one or more individual litter management practices for both surveys.

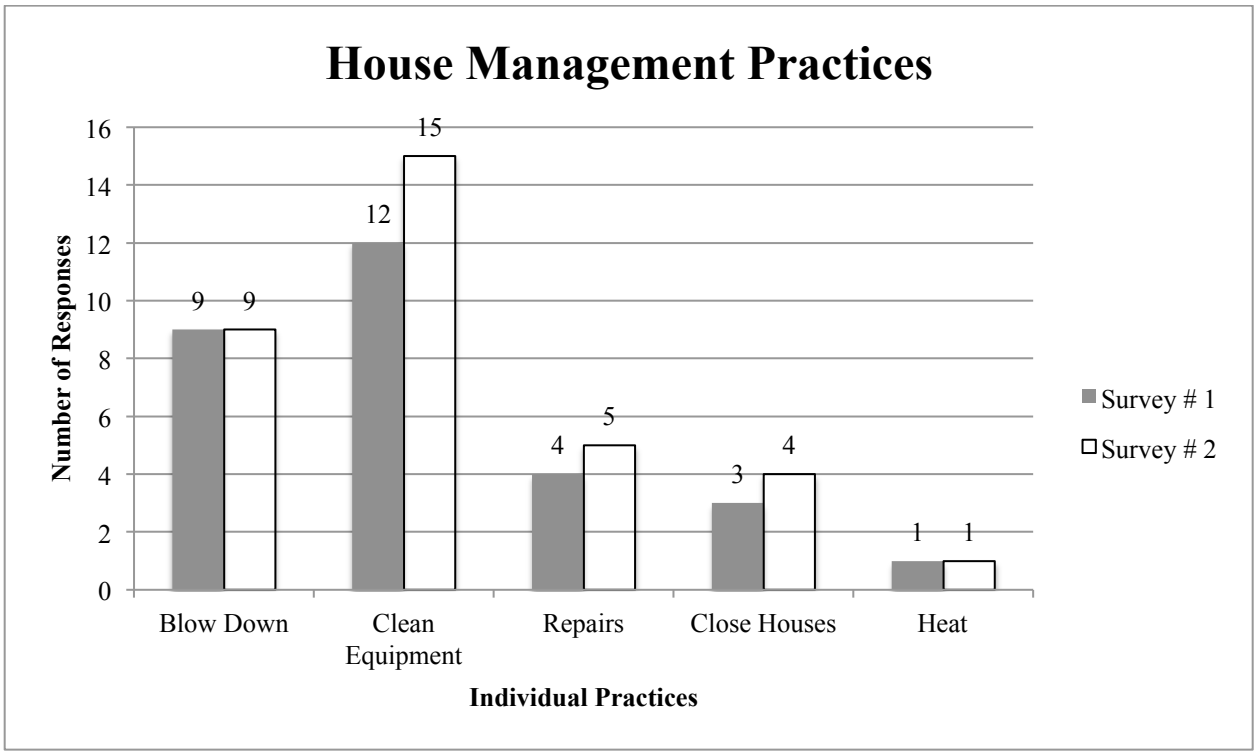

Figure 3.3. Number of responses for individual house management practices for both surveys.

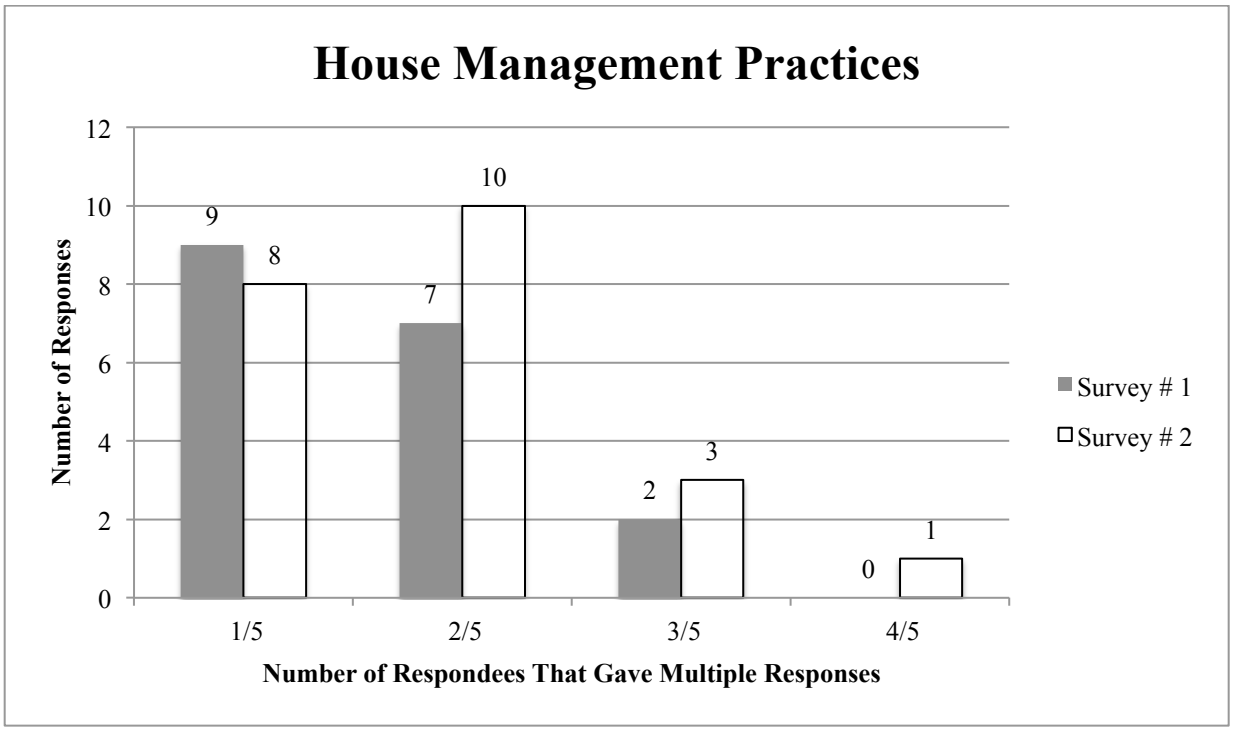

Figure 3.4. Number of responses for house management that indicated they performed one or more individual house management practices for both surveys.

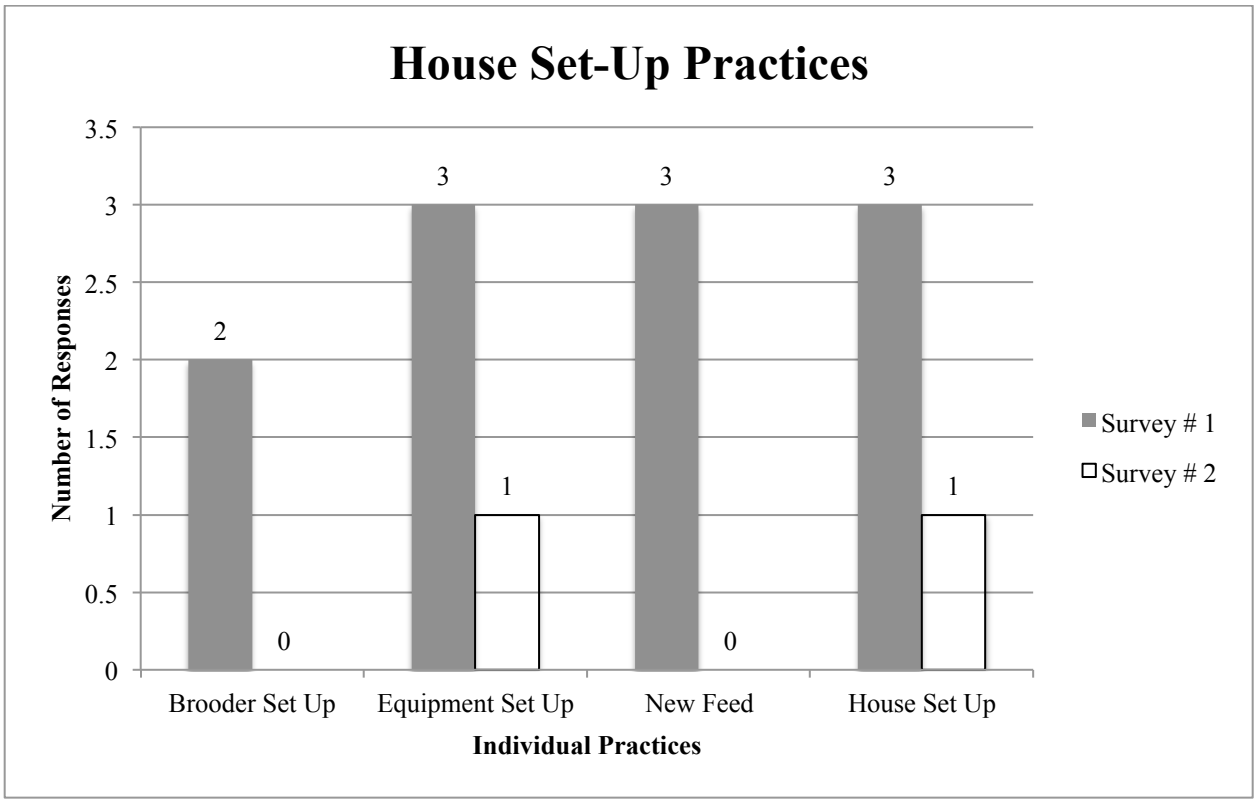

Figure 3.5. Number of responses for individual house set-up practices for both surveys.

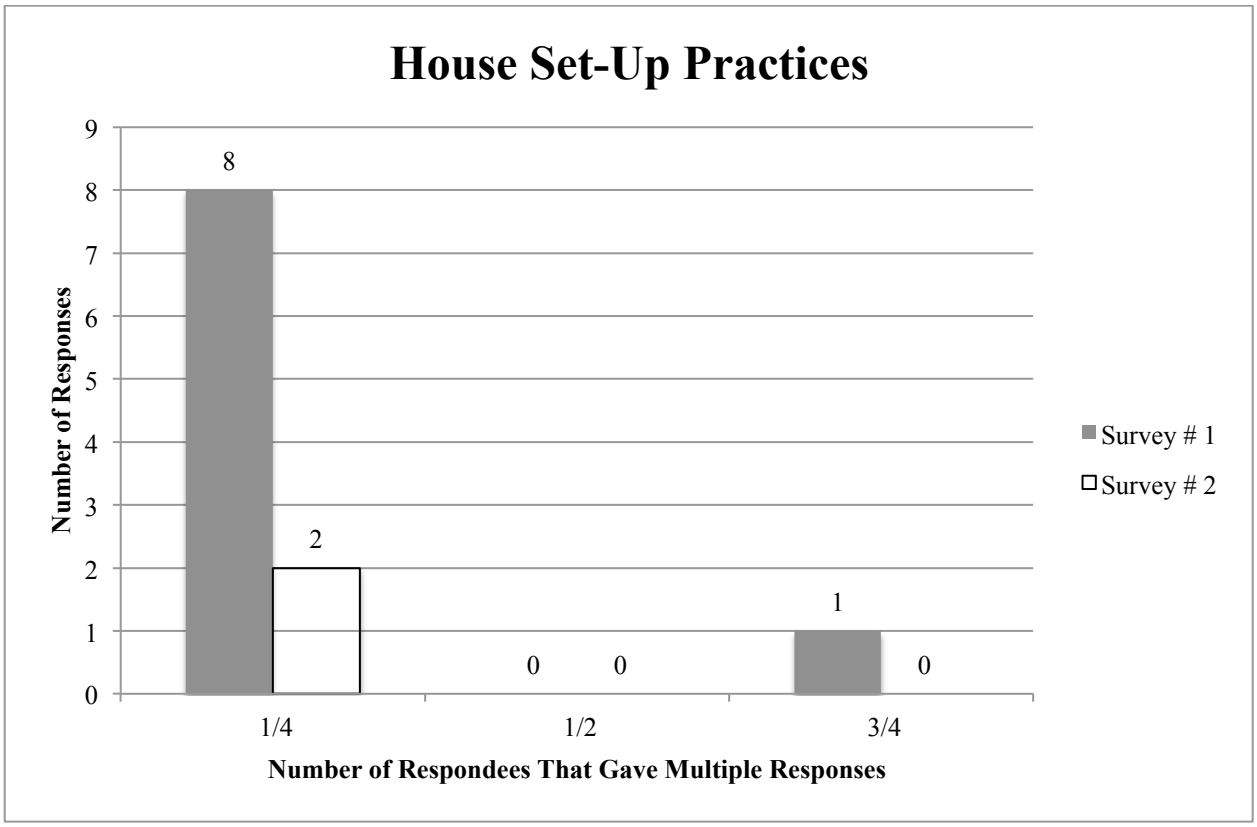

Figure 3.6. Number of responses for house set-up that indicated they performed one or more individual house set-up practices for both surveys.

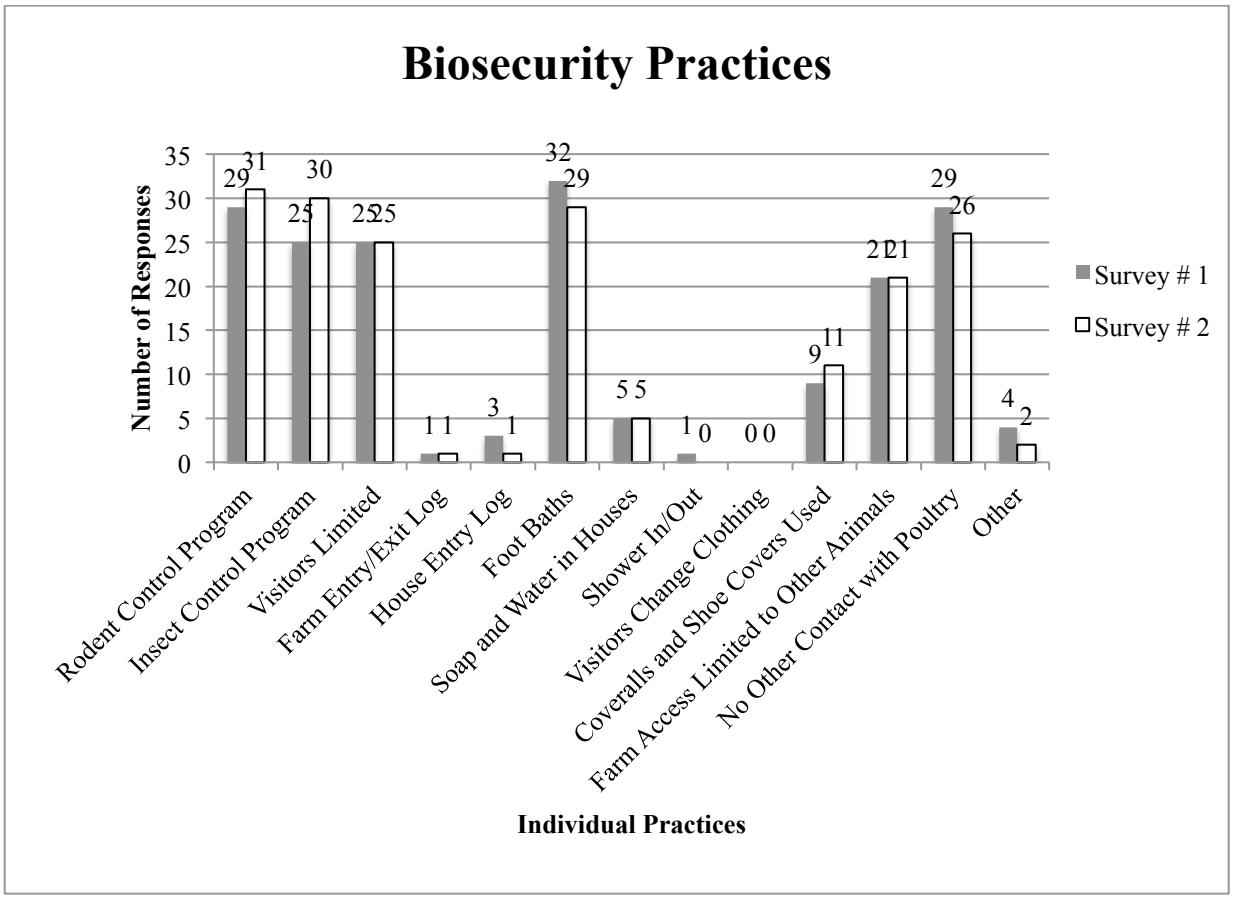

Figure 3.7. Number of responses for individual biosecurity practices for both surveys.

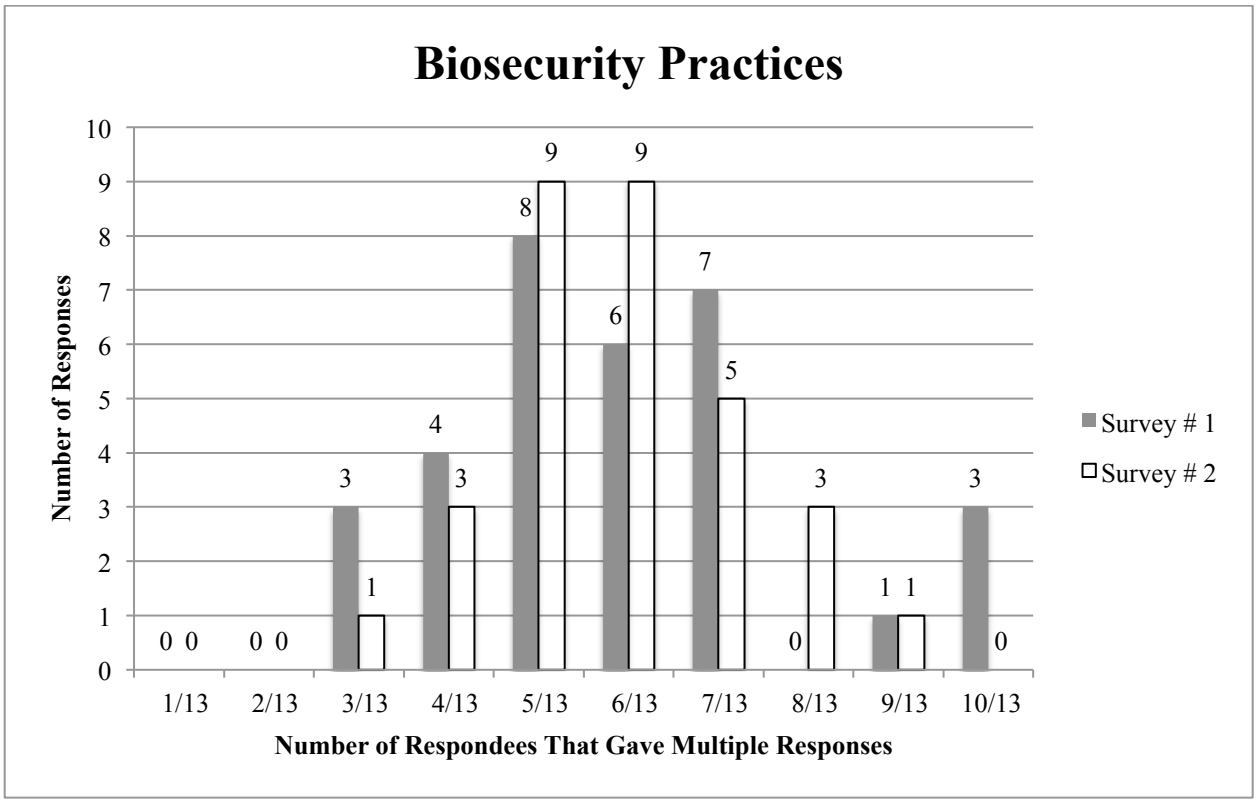

Figure 3.8. Number of responses for biosecurity practices that indicated they performed one or more individual practices for both surveys.

IV. Bacterial Prevalence on Broiler Farms from a Single Integrator in Alabama

ABSTRACT

Recent events have caused the poultry industry to explore alternative methods for microbial control at pre-harvest points during poultry production. Traditionally, the industry has addressed this issue using antibiotics and antimicrobials at post-harvest points in production while assuming that the integrators and growers were adequately addressing biosecurity and management concerns to keep pathogen levels at an acceptable level. However, the recent US Avian Influenza (AI) outbreak, increased regulation on foodborne illness pathogens in poultry meat, restriction on antibiotic use and the subsequent emergence of diseases such as necrotic enteritis (NE) have demonstrated the need for change. The goal of this study was to determine what the baseline microbial population was for four broiler farms in Alabama following basic biosecurity and mangament practices. This was performed by sampling each farm three times at regular intervals throughout one grow out period. During each sampling, cloacal swabs, drag swabs and litter samples were taken and evaluated for *Salmonella, Campylobacter, Clostridium perfringens,* total anaerobic bacteria and total aerobic bacteria. Recommendations were then made to the integrator on biosecurity and management practice improvements and the same farms re-sampled in the same manner to determine if any changes in the microbial community were evident as a result of the implemented changes. Also, a portion of the *C. perfringens* isolates collected during each sampling period were analyzed for the NE associated and toxin producing genes *netB* and *tpeL. Salmonella* and *Campylobacter incidence* ranged from 0-68.75% depending on sample source. *C. perfringens* was recovered at an average of 3.40 logs while total anaerobic bacteria was recovered at an average of 6.63 logs and aerobic bacteria at 8.19 logs. Out of 366 *C. perfringens* isolates examined for the genes *netB* and *tpeL*, only 38

(10.38%) were positive for *netB* and zero were positive for *tpeL*. Overall, there was very little difference between the two sampling periods. This was not unexpected as no evident changes were made to the biosecurity and management practices as a result of our recommendations.

INTRODUCTION

The year 2015 has brought significant changes to the poultry industry in the United States. The regulation changes on the allowable levels of *Salmonella* and *Campylobacter* in raw poultry products has forced the industry as a whole to reexamine the methods currently used for microbial control (Changes, 2015). Also, the midwest outbreak of AI and the subsequent intentional decimation of millions of birds has impacted the industry from an economical and logistics standpoint with effects that will last for years to come. Traditionally, most microbial control efforts have been focused on processing and other post-harvest points while relying on the integrators and growers to maintain acceptable biosecurity and management practices that would allow for the proliferation of pathogens at acceptable levels (Biggs, 1982; Dorea et al, 2010; Bauermeister et al., 2008). However, the recent issues surrounding antibiotic resistance, consumer acceptance of antibiotic use in poultry, the aformentioned regulation changes and widespread AI outbreak, has caused an increased interest in various methods for microbial control at pre-harvest points during poultry production (Fairchild, 2005).

Previous research has repeatedly demonstrated that the poultry house and production environment are a reservoir for bacteria, pathogenic and otherwise (Dumas et al., 2011; Al-Abadi and Al-Mayah, 2012; Dale et al., 2015). Because of this, the production environment is a target for microbial control. One of the most common antimicrobial methods used at the pre-harvest stage is the use of orally administered antibiotics. However, the industry as a whole is in the process of moving away from antibiotic use in poultry, even when disease is present. Because of this reduction in antibiotic use combined with diet formulation changes, the incidence of diseases such as NE caused by *C. perfringens,* are on the rise. For these reasons, the poultry industry has to find alternative methods for microbial control while maximizing profit, consumer

acceptability, efficacy and efficiency. Due to the complexity of the production environment it is often necessary to approach the problem from multiple directions to obtain the desired results. Litter management, breeding, vaccination, general management and biosecurity practices all play a role in maintaining a healthy, pathogen-free flock.

Most recent research has studied the bacteria present in one or more areas of the production environement, the microbial population diversity, and the best methods for obtaining a sample that is a good representation of the microbial population present (McCrea et al., 2005; Lu et al., 2003; Barker et al, 2010; Martin and McCann, 1998; Williams and Macklin, 2013). No conclusive research has been performed that indicates the effect that management and biosecurity practices have on the bacterial prevalence in the production environment. Additionally, recent research on NE has focused on the effect of diet formulation changes, probiotic use, and the novel toxins produced by *C. perfringens* that cause the disease (Keyburn et al., 2010a; Keyburn et al., 2010b; Shojadoost et al., 2012; Williams, 2005; Olivia et al., 2015). Some studies have indicated that the *C. perfingens* genes *netB* and *tpeL* which produce novel toxins are associated with NE incidence in poultry (Keyburn et al., 2010a; Keyburn et al., 2010b; Coursodon et al., 2012; Shojadoost et al., 2012). Other studies have indicated that these genes are not very prevalent in *C. perfringens* isolates and may not be important virulance factors for NE (Bailey et al., 2015; Martin and Smyth, 2009). While previous research has analyzed *C. perfringens* isolates taken from NE positive birds or flocks, this study will examine isolates taken exclusively from litter samples obtained during each sampling visit.

The primary purpose of this study is to produce a consistent bacterial baseline for several farms of a single integrator that have similar characteristics and practices throughout one growout period. In addition, *Salmonella, Clostridium perfringens* and *Campylobacter* incidence

will be evaluated and compared in order to potentially identify sources of contamination and bacterial hotspots within each house. This will be performed by regularly visiting each farm, taking litter samples, drag swabs and cloacal swabs that will then be analyzed to produce a bacterial baseline. Then, biosecurity and management recommendations will be submitted to the integrator and the same farms re-sampled in the same manner to determine if there were any observable changes in the microbial population as a result of the biosecurity and management changes. The second objective of this study is to analyze a portion of the *C. perfringens* isolates taken from the litter samples for the presence of the genes *netB* and *tpeL* in order to contribute to the current knowledge base on the prevalence of these two genes in the normal production environment.

MATERIALS AND METHODS

Experimental Design

Four farms from the same integrator were selected for participation in this study based on the average farm characteristics found in the biosecurity and management survey sent several months previous to sample collection. Each farm had chicks placed within one day of each other and was visited three times (March – April) at equal intervals (D1,14 and 30; +/- 1D), labeled visits 1A-1C, throughout one grow-out period. After the initial survey was distributed, responses collected and analyzed. Biosecurity program improvement recommendations were made after the conclusion of the sampling period. Nine months after the first survey was sent, the same farms were resurveyed and subsequently sampled in the same manner as before (Visits 2A-2C), except during the fall (October-November). Each farm was visited a total of six times over a span of 9 months.

Farm Characteristics

Each poultry farm had four houses of the same size, three farms had 40x400 ft houses and the remaining farm had 40x500 ft houses. Two farms had conventional style housing, one farm had tunnel ventilated housing and the remaining farm had two tunnel ventilated houses and two conventional style houses. Three farms had drop curtains and the remaining farm was of solid wall construction. All farms had litter (pine shavings) approximately the same age (1 year) and was completely removed after the first sampling period. Three farms had front end split brooding arrangements, while the fourth farm had the front half of each houses sectioned off for brooding. Three farms top dressed the litter prior to chick arrival and two farms applied a litter acidifying treatment as well (Table 4.1).

Sample Collection

Upon entry to each farm, each person from AU donned disposable coveralls, plastic shoe covers, disposable gloves, hair nets, breathing masks and protective eyewear. Gloves and shoe covers were changed in between each house and all equipment except eyewear was discarded in between farms. In addition, the farms provided footbaths that were also utilized before entering and exiting each house.

All samples were collected and processed in the laboratory within a twenty-four hour period for all sampling visits. Three types of samples were taken: cloacal swabs, litter samples and drag swabs. Twelve cloacal swabs per house were taken from birds at random using sterile polyester tipped applicators (product no. 25-806 1PD; Puritan, Guilford, ME) (n = 192 per sampling). Each polyester applicator was premoistened with buffered peptone water (BPW) (product no. C5323; Criterion, Santa Maria, CA) before swabbing the cloaca, and then inoculated into a sterile glass test tube that had 5 ml of BPW, and was used to transport the

samples back to the laboratory. All cloacal samples were kept on ice in insulated coolers after collection until processing.

Four litter samples were taken per house (n = 64 per sampling). Each house was divided into four quadrants across the width of each house. Samples were collected in each quadrant by alternating sides of the house (first sample was taken on the left side of the first quadrant, second sample was taken on right side of second quadrent etc.). Each litter sample was composed of three subsamples of approximately 100 grams each, and were taken between the first water line and the side of the house, between the two water lines and around the feed line, and lastly between the second water line and the middle of the house. The litter samples were taken in this method to ensure an accurate representation of that section of the house (Figure 4.1). The three litter subsamples were combined and mixed thoroughly by hand in one gallon plastic bags, and were mixed again prior to processing in the laboratory.

Eight sterile drag swabs (product no. DS-004; Solar-Cult, Ogdensburg, NY) were taken per house (n = 128 per sampling), four were for *Salmonella* enrichment and four were for *Campylobacter* enrichment. Similar to the litter sampling described above, each house was divided into four quadrants. Starting from the middle of the house, two drag swabs were dragged between the water line and house wall until the end of the house was reached. After rounding the feed and water lines, the same swabs were then dragged between the second water line and the midline of the house until the middle of the house was again reached (Figure 4.1). Each drag swab was then placed back in its container until processing could occur.

Enrichment and Plating Procedures

Drag swabs and cloacal swabs were both selectively enriched for both *Campylobacter* and *Salmonella*. Half of the drag swabs from each house had 20 mls of Bolton's Broth (product

no. 7526A; Campylobacter Enrichment Broth, Acumedia, Baltimore, MD) added and were then vortexed to mix thoroughly before incubating under microaerophilic conditions (5% CO_2, 5% H_2 and 90% N_2) at 42° C for 24 hours. The other half of the drag swabs had 20 mls of Tetrathionate Brilliant Green Bile Broth (TTB) (product no. 95020-798; Himedia, Mumbai, India) added and were then vortexed to mix thoroughly before incubating at 37°C for 24 hours. From the cloacal swab BPW tunes 1 ml was taken and aliquoted into tubes that contained either 20 ml TTB or 20 ml Bolton's Broth so that each swab was enriched for both *Salmonella* and *Campylobacter* in the same manner as mentioned above.

After incubation, the Bolton's Broth cultures were streaked onto Campy Cefex agar plates (product no. 7718A; Acumedia, Baltimore, MD) which were then incubated under microaerophilic conditions at 42°C for 24 hours. Suspect *Campylobacter* colonies were suspended in sterile water on a microscope slide with a coverslip and examined under a phase contrast microscope. Colonies that exhibited a spiral morphology were cultured in a Bolton's broth solution and then preserved in cryovials and frozen at -80°C for future analysis as described by Gorman and Adley (2004).

After incubation, the TTB cultures were streaked onto xylose lysine tergitol 4 (XLT4) agar plates (product no. 223420; BD, Sparks, MD) and incubated at 37° C for 48 hours. Suspect *Salmonella* colonies (black) were tested with an antiserum (Difco™ Salmonella O Antiserum Poly A – I and Vi; product no. 222641, BD, Sparks, MD) and if the solution precipitated, the colony was confirmed as *Salmonella*. Confirmed *Salmonella* isolates (4 isolates per sample when possible) were cultured in brain heart infusion (BHI) Broth (Bacto™ Brain Heart infusion, product no. 237500, BD, Sparks, MD) with 20% glycerol and then frozen in cryovials on sterile glass beads at -80°C for future analysis.

Litter samples were processed by putting 10 grams of each litter sample into 15.2x22.9 cm filter bags (VWR Sterile Sampling Bag, product no. 89085-570, VWR) to which 90 ml of phosphate buffered saline (PBS) was added. Each bag was then stomached for 60 seconds and then serially diluted using PBS. This solution was then serially diluted (1:10) and 100 µl of each diluted solution was spread plated onto anaerobic agar (product no. 253610; BD, Sparks, MD), plate count agar (PCA) (product no. 247940; BD, Sparks, MD), and tryptose sulfite cycloserine (TSC) agar (product no. VM647372 429; Merck kGaA, Germany). The anaerobic agar plates were incubated in a Bactron IV Anaerobic Environmental chamber (Shel Lab, Cornelius, OR) at 40°C for 48 hours to cultivate the anaerobic organisms present in the sample, and then individual colonies were counted on the plates that had between 30-300 individual colonies present. The PCA plates were incubated at 37°C for 24 hours to cultivate the aerobic organisms present in the sample and were then enumerated following the same procedure. The TSC plates were also incubated in the chamber at 40° C for 48 hours to cultivate *Clostridium perfringens*. Colonies indicative of this organism (black) were then enumerated using the same procedure already described. Several samples did not exhibit any black colonies so 5 grams of poultry litter was stomached with 45 ml of PBS and 500 µl of this solution aliquoted into tubes with 10 ml of cooked meat broth (product no. C5481; Criterion, Santa Maria, CA) and incubated at 40°C in the Bactro IV Anaerobic Environmental Chamber for 24 hours. The cooked meat enrichment culture was then streaked onto TSC plates and incubated in the same manner as before to detect if *C. perfringens* was present in the original sample. A portion of the black colonies (4 per sample when possible) from the TSC plates were isolated, cultured in BHI broth with 20% glycerol, and then frozen in cryovials on sterile glass beads at -80°C so that they could later be confirmed as *C. perfringens* and analyzed for specific genes.

Polymerase Chain Reaction (PCR)

One *C. perfringens* isolate per litter sample (when available) was randomly chosen for genetic analysis by PCR. Each *C. perfringens* isolate was grown by putting a single glass bead from the cryovial onto a Trypticase Soy Agar with 5% Sheep Blood plate (TSA II) (BD, Sparks, MD) which was then incubated in a Bactron IV Anaerobic Environmental Chamber at 37°C for 24 hours. A single colony was chosen and inoculated into 5 mls of BHI and then incubated in the anaerobic chamber at 37°C for 24 hours. The DNA from each *C. perfringens* isolate was then extracted using a Wizard® Genomic DNA Purification Kit (product no. A1125, Promega Corp., Madison, WI). The extracted DNA was then analyzed using 1 μL of each DNA extraction using a Nanodrop 1000 Spectrophotometer (Thermo Scientific Inc., Bremen, Germany) for DNA concentration and sample purity using the 260/280 ratio with the a desired value between 1.8-20 (Bailey et al, 2013)

The primers used in this study were based on those used in a previous study to detect the genes *netB* and *tpeL* in *C. perfringens* (Table 4.2.) (Bailey et al., 2013; Keyburn et al., 2010). The same reagent concentrations were used for each reaction and consisted of the following: 1X of EconoTaq® PLUS GREEN Master Mix (Lucigen, Middleton, WI, no. 30033-1), at 12.5 μl per reaction. To this was added 0.125 μl of each forward and reverse primers (1X), 5 μl of extracted DNA and 7 μl of water (25 μl total per reaction). DNA amplification was then performed by Multiplex PCR by using an iQ5 thermocycler (Bio-Rad, Hercules, CA). The PCR cycle parameters were as follows: 95° C for 5 minutes (denatures DNA), then 40 cycles of 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds (annealing and extension). The reactions were then held at 72° C for six minutes and lastly at 4° C prior to removal from the thermocycler.

The PCR products were then separated by gel electrophoresis using a 1.5% agarose gel (Agarose, OmniPur, no. 2120, Gibbstown, NJ). The gels and buffer used during analysis was made from 1X Tris-Acetate-EDTA (TAE) buffer (Bio-Rad, no. 161-0743, Hercules, CA). Each gel had 4 µl of ethidium bromide (10mg/mL) added prior to solidification to allow the PCR product bands to be observed. The gel was then run at 75v for approximately one hour to allow for the PCR products to separate. A DNA ladder (100 bp, Promega, Madison, WI) was used as a size standard for the PCR products. A negative control (pure water) and a positive control (*C. perfringens* isolate previously confirmed as *netB* and *tpeL* positive) were used during each gel electrophoresis run. The *netB* amplicon bands from gel electophoresis were excised and then purified and sequenced (6 total) to confirm that these isolates indeed have the *netB* gene amplified. A nucleotide BLAST search was performed using the sequences obtained from the purified *netB* positive PCR bands. The top results from the BLAST search that had a minimum of 90% identity with the searched sequence were considered relevent. A small E-value for each search result also indicated that the top results were matches for the searched sequences.

Data Analysis

All data, including PCR results was analyzed using SPSS (version 22.0.0.0) and General Linear Model (GLM), using Tukey HSD to separate the means using $\alpha = 0.05$. A p-value ≤ 0.05 was considered statistically significant. Bacterial counts (*Clostridium perfringens,* anaerobic and aerobic bacteria) were log transformed. *Salmonella* and *C. perfringens* percentage results were arcsine transformed. Arcsine transformation results did not improve the the data distribution so data is displayed as percentages.

RESULTS

Cloacal Swabs

Figure 4.2 exhibits the results by visit and farm for the cloacal swabs that were enriched for *Salmonella.* There was no statistical significance between the farms for the first sampling period (Visits 1A-1C). For the second sampling period (Visits 2A-2C), only visit 2A showed statistical significance between farms ($p \leq 0.05$). The percent positive samples from Farm 1 (18.75%) for visit 2A was significantly different than the results from Farm 4 (0.00%). When comparing the two first visits (1A and 2A) for both sampling periods, visit 2A has a much higher overall incidence of *Salmonella* compared to visit 1A. Visit 2A had a maximum of 18.75% incidence with an average recovery of 9.90%. Visit 1A had a maximum of 10.42% incidence with an average recovery of 6.25%. The two middle visits (1B and 2B) followed the same trend as the first visits, with visit 2B exhibiting a much higher incidence of Salmonella (9.38% average) than visit 1B (4.17%). The last visits from both sampling periods were more similar to each other than for the previous visits. Visit 1C had an average incidence of 6.25% while visit 2C had an average of 5.21%. The average percent positive samples for the first sampling period was 5.56% and 8.16% for the second sampling period. The average percent positive samples for both sampling periods was 6.86%. The minimum percent positive samples observed per farm was 0.00% and the maximum percent positive samples observed per farm was 18.75%.

Ther results for the cloacal swabs enriched for *Campylobacter* are displayed in Figure 4.3. There was no statistical significance for the first sampling period and only two farms (Farms 2 and 4) had positive samples (2.08% each). All other farms during the first sampling period did not any samples positive for *Campylobacter*. For the second sampling period, the first visit (2A) had no positive samples for any farm. Visits 2B and 2C were both statistically significant ($p \leq 0.05$). Farm 1 (27.08%) for visit 2B was statistically significant compared to the other farms that had 0.00% (Farms 2 and 4) and 6.25% (Farm 3). For visit 2C, Farm 4 (68.75%) had a

statistically significant higher incidence of *Campylobacter* compared to the other three farms that had results ranging from 14.58%-37.50%. The minimum percent positive samples observed per farm was 0.00%, and the maximum percent positive samples observed per farm was 68.75%.

Drag Swabs

Figure 4.4 shows the results by visit and farm for the drag swabs that were enriched for *Salmonella*. There was no statistical significance for any visit or farm over the two sampling periods ($p \geq 0.05$). Overall, except for the visit 2C, the *Salmonella* incidence was very similar between all visits. During the second sampling, three farms had 0% recovery whereas the first sampling only had one case of 0% recovery. For both visits 1A and 1B, the individual *Salmonella* incidence measured by each farm remained the same for both visits. Both visits 1A and 1B had the same recovery rate of 15.63% while both visits 2A and 2B also had a similar recovery rate of 14.06%. The last visits exhibited the most difference between sampling periods. Visit 1C had an average recovery of 17.19% while visit 2C had an average recovery of 46.88%. The average percent positive samples for the first sampling period was 16.15% and 25.00% for the second sampling period. The overall average percent positive samples was 20.57%. The minimum percent positive samples observed per farm was 0.00%, and the maximum percent positive samples observed per farm was 68.75%. Figure 4.5 displays the results by location for the drag swabs enriched for *Salmonella* during Visit 2A. Location 1 (37.5%) had a statistically significant higher incidence of Salmonella then Locations 3 (0.00%) and 4 (6.25%). There was no statistical significance by location for any of the other visits over the two sampling periods. There were no positive samples for the drag swabs enriched for *Campylobacter* for either sampling periods.

Litter Samples

There was no statistical significance for any visit or farm over the two sampling periods for the litter samples enriched for *Salmonella* ($p \geq 0.05$) (Figure 4.6). Both first visits had the overall lowest incidence of *Salmonella.* For visit 1A, three of the four farms had 0% recovery while the remaining farm had a 6.25% incidence (1.56% average). Visit 2A had three farms that had a 6.25% incidence and one farm that had 0% recovery (4.69% average). The recovery rate increased over the second samplings but were still sporadic with visit 1B having two farms with 0% recovery (10.94% average) and visit 2B having an average of 14.06% incidence with one farm showing 0% recovery. The last visits were more consistent with all farms reporting positive samples, however visit 1C had an overall lower recovery (12.5% average) while visit 2C had a much higher recovery (21.88% average).The average percent positive samples for the first sampling period was 8.33% and 13.54% for the second sampling period. The overall average percent positive samples is 10.94%. The minimum percent positive samples observed per farm was 0.00%, and the maximum was 31.25%.

The *Clostridium perfringens* results recovered from the litter samples is displayed in Figure 4.7 with the results expressed in log10 CFU. For the first sampling period, only Visit 1A was not statistically significant ($p \leq 0.05$). Farm 4 (4.03 logs) had statistically significant more *Clostridium perfringens* then Farms 1 (3.07 logs) and 3 (3.27 logs) for Visit 1B. Farm 1 (3.82 logs) had statistically significant higher *Clostridium perfringens* counts then Farms 3 (2.82 logs) and 4 (2.99 logs) for Visit 1C. For the second sampling period, only Visit 2C was not statistically significant. Farm 4 (3.77 logs) had statistically significant higher *Clostridium perfringens* counts then Farm 3 (3.19 logs) for Visit 2A. Farm 4 (3.62 logs) had statistically significant higher bacteria counts then Farms 1 (3.09 logs) and 3 (3.03 logs) for Visit 2B. The average number of log10 CFUs recovered during the first sampling period was highest at the first visit (3.61 logs)

and steadily decreased until the last visit (3.22 logs). The second sampling period followed the same trend for the first two visits, starting with an average of 3.42 logs and decreasing to 3.27 logs on the second visit. The last visit exhibited a slight uptick with an average of 3.43 logs. Comparisons of the first, middle and last visits for both sampling periods had about 0.2 logs difference between each visit. Visit 1A had an average of 3.61 logs while visit 2A had 3.42 logs. The middle visits were slightly lower with visit 1B having 3.45 logs and 2B 3.27 logs. The only instance where the second sampling period had a higher level of *C. perfringens* than the first sampling period was during the last visit. Visit 1C had an average of 3.22 logs while visit 2C had an average of 3.43 logs.The average number of log10 CFUs of *C. perfringens* recovered from litter during the first sampling period was 3.43, while for the second sampling it was 3.37. The overall average number of log10 CFUs of *C. perfringens* recovered is 3.40. The minimum of log10 bacteria recovered was 2.82 and the maximum number recovered was 4.03.

Figure 4.8 displays the results in log10 CFUs by location for the *C. perfringens* recovered from litter samples during Visit 2C. Location 4 (3.64 logs) had significantly more *Clostridium perfringens* then Location 3 (3.16 logs). There was no statistical significant difference by location for any other visits over the two sampling periods.

Three of the six sampling visits were statistically significant for the recovery of anaerobic bacteria (Figure 4.9). During the first sampling period, Visits 1A and 1B had statistically significant ($p \leq 0.05$) differences. Visit 1A, Farm 1 (6.59 log10 CFUs) had significantly higher anaerobic counts then Farms 3 (5.39 logs) and 4 (5.70 logs). For Visit 1B, Farm 4 (8.00 logs) had significantly higher counts then Farms 1 (7.48 logs) and 2 (7.54 logs). Only Visit 2B showed any signicant differences for the second sampling period. Farm 4 (7.65 logs) was statistically different from Farms 1 (7.18 logs), 2 (7.21 logs), and 3 (6.49 logs). It was also determined from

that visit that Farm 3 had statistically significant lower bacterial counts than Farms 1 and 2. The minimum number of log10 CFUs observed for all visits was 4.94, and the maximum number was 8.00. The average log10 CFU of anaerobic bacteria recovered for the first sampling period was 6.77, and 6.48 for the second sampling period. The overall anaerobic bacteria average recovered for all visits was 6.63.

Figure 4.10 displays the results by location for the number of log10 CFUs of anaerobic bacteria recovered from litter samples during Visit 1A. Location 1 (6.35 logs) had significantly more anaerobic bacteria then Location 3 (5.51 logs). The anaerobic bacteria results from the litter samples, by location for visit 2B is exhibited in Figure 4.11. As can be observed in this table, Location 4 (6.55 logs) was significantly different from Locations 1 (7.42 logs), 2 (7.42 logs) and 3 (7.13 logs). There was no statistical difference by location for any other visits over the two sampling periods.

For the aerobic bacteria recovered from litter samples, three of the six visits were statistically significant (4.12 log10 CFUs). During the first sampling period, both Visits 1A and 1C were statistically significant ($p \leq 0.05$). Farms 1 and 2 had significantly higher bacteiral counts (7.91 logs and 8.03 logs respectively) then Farm 3 (6.43 logs) and Farm 4 (6.77 logs) for Visit 1A. Farm 4 is also significantly different from Farm 3. For Visit 1C, Farms 1 (8.82 logs), 2 (9.24 logs) and 4 (8.90 logs) had significantly higher aerobic bacterial counts then Farm 3 (8.26 logs). Only visit 2A was statisticallly significant for the second sampling period. Farm 2 (8.25 logs) had significaly different bacterial counts then Farms 1 (7.39 logs), 3 (6.74 logs) and 4 (7.36 logs). Similar to visit 1A, Farm 3 was also significantly different from Farm 4. The average number of log10 bacteria CFUs recovered from the first sampling period was 7.91, and 8.46 for the second sampling period. The overall average number of bacteria recovered over both

sampling periods was 8.19. The minimum number of log10 CFU of aerobic bacteria recovered from litter samples for both sampling periods was 6.43. The maximum number recovered was 9.24 logs.

Figure 4.13 displays the results by location for the number of aerobic bacteria recovered from litter samples during Visit 1B. Locations 1 (9.50 log10 CFUs) and 2 (9.35 logs) were significantly different from Location 4 (8.87 logs). Figure 4.14 displays the results by location for the number of logs of aerobic bacteria during Visit 2B. Locations 1 (9.20 logs) and 2 (9.49 logs) were significantly higher bacterial counts then Location 4 (8.23 logs). There was no statistical significance by location for any other visits over the two sampling periods.

PCR Results

Of the 366 suspect *C. perfringens* isolates analyzed by PCR for the genes *netB* and *tpeL*, 38 (10.38%) were positive for *netB* and zero isolates were positive for *tpeL* (Table 4.3). Only visit number one was statistically significant for the incidence of *netB* positive *C. perfringens* isolates (Figure 4.15). There was no statistical significance by farm or location for the incidence of *netB* positive isolates. However, it is noteworthy to mention that location 3 was close to being significantly different to location 2 (p-value = 0.055). Figure 4.16 shows several examples of isolates positive for *netB*. BLAST search results indicated that all sequenced PCR products matched the target gene with a high degree of certainty (Table 4.4). From this it is evident that PCR accurately identified the *netB* positive isolates.

DISCUSSION

Pre-harvest control points are the next big target for pathogen reduction in poultry production due to increased regulation of *Salmonella* and *Campylobacter* levels allowed on raw poultry products. Because of these stricter regulations and the midwest AI outbreak, the poultry

industry has renewed interest in the effect that biosecurity and management practices have on the prevalence of pathogens, specifically pathogens in the poultry production environment. There is not a universal, industry wide biosecurity program practiced by all integrators across the industry. Instead, each integrator is responsible for developing and enforcing their individual biosecurity programs. Because of this there is great variability in compliance and efficacy between farms and integrators. This leads to inconsistent practices across the industry and allowing for the spread and proliferation of diseases such as NE. Since biosecurity is the responsibility of the farmers and integrators, it is important that they be willing to adjust their biosecurity and management programs as needed to ensure full coverage that would prevent the spread of disease. As the results from this study indicate, farmers and integrators are not always willing to change their biosecurity and management programs, mainly for the lack of immediate return on investment. Our on farm observations and conversations with the poultry farmers indicated that the integrator made no effort to change their biosecurity and management program. This was further demonstrated by the overall lack of statistical significance between the two sampling periods, especially for the pathogens *Salmonella, Clostridium perfringens*, and *Campylobacter*. For the cloacal swabs, only one visit was statistically significant when evaluating *Salmonella* incidence, and two visits for *Campylobacter* incidence. The drag swabs did not exhibit any statistical significance for any visit for either *Salmonella* or *Campylobacter.* However, one location within the houses was significant for *Salmonella* incidence. The litter samples did exhibit more differences overall than the two other methods of sample collection. However, *Salmonella* did not exhibit any difference. *Clostridium perfringens,* anaerobic and aerobic bacteria all showed several cases of statistical significance by visit, farm, and location.

While overall there was not a lot of statistical significance, there were many instances where there were observable differences.

There was only one visit that was statistically significant for *Salmonella* incidence from cloacal swabs, and it is obvious from Figure 4.2 that the overall levels of *Salmonella* where highly variable across all six visits. However, the second sampling period overall had a higher incidence of *Salmonella*. The first visits for both sampling periods had moderate to high numbers of positive samples when compared to the other visits. This is important to note as the birds were sampled within 36 hours from when they were hatched. This implies that they were contaminated prior to placement at the farm, most likely from the hatchery or breeder flock, and potentially act as a source of contamination for future flocks raised in the same facilities. Conversely, the last visits for each sampling period also had moderate to high numbers of positive samples. This is important as these samples were taken only a few days prior to when the flocks were sent to the processing plant, indicating that these birds were positive for *Salmonella* when entering the plant and possibly acting as a source of contamination for all the other birds passing through the same facility. Lastly, important to note is that only 12 cloacal swabs were taken from each house on each visit. According to the survey sent prior to sampling, the average number of birds on each farm is 77,855 divided into 3.3 houses (23,600 birds per house) This means that only 0.0005% of the birds in each house had cloacal swabs taken. This is a very small number compared to the thousands of birds present in each house. Given this fact, the recovery of up to 18.75% positive samples per house could actually be much higher if a larger sample set were taken. Previous cloacal swab studies (for broiler and layer flocks) have had inconsistent findings that ranged from 2% *Salmonella* recovery (Balala et al., 2006) to 19% as found by Al-Abadi and Al-Mayah (2012). The results from this study (6.86% average) is

consistent with these studies, and the percentage range published previously (Garcia et al., 2011; Al-Abadi and Al-Mayah, 2012; Balala et al., 2006). The difference in recovery rate could be due to different methods used to house layers and broiler flocks. Two previous studies included layer flocks in their analysis while our study collected samples exclusivley from broiler flocks (Garcia et al., 2011; Al-Abadi and Al-Mayah, 2012).

There were only two positive samples for *Campylobacter* from the cloacal swabs taken during the first sampling period. The lack of positive samples was attributed to the isolation and culture methods used during this period. After re-evaluating the culture methods used, it was determined that after sample collection, the samples should be kept under microaerophilic conditions while being cultured. Recovery rate of *Campylobacter* increased over the second sampling period, with visits 2B and 2C being statistically significant. While the recovery rate did increase when the culture methods were changed, the findings were still highly vairable with values ranging from 0-68.75%. Previous studies found that 17.8% of cloacal swabs were positive for *Campylobacter,* and 28% of litter surface evaluations by boot sock were positive for the organism (Fonseca et al., 2006; Dale et al., 2015). The few positive *Campylobacter* samples from this study could be due to farm management and biosecurity practices, seemingly poor survivability of the organism in the environment, farm location and type of farm. It is important to note that the highest recovery rate occurred on the very last visit indicating that the birds were contaminated at the end of their grow-out period and when they entered the processing facility (Figure 4.3). The recovery of *Campylobacter* from cloacal swabs in previous studies is highly inconsistent. Alves et al. (2012) found 9.4% of samples were positive for this organism while Fonseca et al. (2006) found 17.8%. Other studies found levels ranging from 41.0 – 72.2% (Vaz et al., 2012; Stern and Robach, 1995). While the recovery of this organism in this study was not

an accurate measure of the contamination levels on the farms, the average recovery for the last visit (2C) was 35.94% which falls into the middle of the percentage range of values found by previous studies.

There was no statistical significance by farm or visit for the *Salmonella* recovered from the drag swabs over both sampling periods. However, the recovery rate was fairly consistent for the first three visits, and sporadic for the last three visits. The very last visit exhibited the highest recovery rate and similar to the observations for the cloacal swabs, this indicates that the flocks were contaminated with *Salmonella* when they entered the processing facility. For visit 2A, location was statistically significant with both quadrants at the rear of the house being significantly different, with higher levels of *Salmonella* than one of the front quadrants and sharing some similarity with the other front quadrant. This could be because there is usually more activity at the front of the house when the chicks are being unloaded from the hatchery and are therefore more likely to be exposed to pathogens. In addition, several farms brooded their chicks in the front half of the house which could also contribute to the higher levels of *Salmonella* found at the front of the house if the chicks were positive for the organism at the hatchery and served as a source of contamination for the litter. Previous studies have found between 2.1-76.2% recovery of *Salmonella* (Caldwell et al., 1994; Byrd et al., 1997; McCrea et al., 2005). The average recovery of 20.57% found in this study is consistent with these previous findings. No drag swabs were positive for *Campylobacter* over either sampling period despite the change in culturing protocol. This could possibly indicate that drag swabs are not always an effective method for recovering *Campylobacter* from poultry litter material. This is confirmed by Stern and Robach (1995) who found that of three sample collection methods (fecal droppings, cecal droppings and cloacal swabs), cecal droppings were the best method to use when an

evaluation of *Campylobacter* levels in poultry production is desired. Several other studies found results conflicitng with Stern and Robach's (1995) findings. One study found that drag swabs that were enriched by using selective media resulted in lower numbers of positive samples (24.07%) than if they were plated directly (66.67%) (Vaz et al., 2012). Another study found that an average of 60.6% of drag swabs were positive for *Campylobacter* (Berghaus et al., 2013).

Salmonella recovery from the litter samples over both sampling periods was sporadic over all six visits, with seven different occurrences of zero percent recovery and two instances of over 30% recovery. Despite this variance, there was no statistical significance for any visit, farm or location over both sampling periods. This study found that 10.94% of all litter samples collected over both samping periods were positive for *Salmonella.* Previous studies have found varying levels of litter samples positive for *Salmonella* with values ranging from 0-100% (Chen and Jiang, 2014; Al-Abadi and Al-Mayah, 2012;Martin et al., 1998). The variable results from our and previous studies indicate that *Salmonella* levels can fluctuate in poultry litter and is perhaps not the most reliable method for determining the presence of this pathogen in a poultry production environment

A lot of research has been performed to determine which method of *Salmonella* detection in litter is most accurate and efficient, drag swabs, shoe covers or litter samples. Two studies found that shoe covers were more effective at accurately detecting *Salmonella* (Buhr et al., 2007; McCrea et al., 2005) while another study found that drag swabs were more effective at recovering *Salmonella* than litter samples (Kingston, 1981). In addition, Buhr et al. (2007) also determined that both drag swabs and litter samples were less accurate than shoe covers were at detecting this pathogen. The difference in recovery for drag swabs between our study and those performed previously could be due to several factors including farm location and pathogen load

at the time of samplingWhile previous research does suggest that drag swabs are more accurate than litter samples, both methods of sample collection were used in this study for comparison to previous studies. Our findings on the evaluation of *Salmonella* on the litter surface were similar to previous studies despite the use of drag swabs over shoe covers.

Recovery of *C. perfringens* from the litter samples was fairly consistent with values ranging from 2.82 to 4.03 log10 CFUs. Four of the six visits had statistically significant differences. The number of log10 CFUs for the first visits was consistent with those for the other visits, probably due to the litter being approximately a year old with an established microbial population. Despite the addition of the chicks, the values recovered from the litter remained fairly consistent. Several studies have examined poultry litter for the presence of *C. perfringens* . One study found that 7.78% of rDNA sequences isolated from poultry litter share homology with Clostridial species, but they determined that they were from uncharacterized species (Lu et al., 2002). Another study found only 1.6 logs of *C. perfringens* present in the upper third of the litter column (Williams and Macklin, 2013). The results of this study had much higher levels of this organism than that found by Williams and Macklin (2013).

The total anaerobic bacteria recovered from the litter samples was statistically significant for visits 1A, 1B and 2B. Overall, The first visits exhibited lower numbers of anaerobic bacteria (5.56 logs on average) compared to the other visits (6.97 logs on average). This could possibly be attributed to most of the houses top dressing their litter with new shavings prior to new chick placement and this process diluting out the numbers. The new shavings would not have the same microbial population as the existing litter due to a lower moisture content, increased exposure to oxygen due to less compaction and the intact physical integrity of the shavings that had not been broken down like the existing litter. Location for visits 1A and 2B was significant for total

anaerobic bacteria with both front of house quadrants having higher levels compared to the rear of the house. However, for visit 1A only the front left and rear left quadrants were statistically significant with the front quadrant exhibiting 6.35 log10 CFU of anaerobic bacteria and the rear quadrant exhibiting 5.51 logs. The higher bacterial levels at the front of the house could again be attributed to front house brooding and the increased litter moisture that occurs as a result. The rear left quadrant with the lowest levels of anaerobic bacteria could be attributed to the fact that it is the farthest from an entrance (located at front or one side of house) and therefore gets less traffic. The results from visit 2B were similar however only the rear right quadrant was significantly different and had reduced levels of anaerobic bacteria compared to the other quadrants. Also important to note, *C. perfringens* is included in the recovery of anaerobic bacteria and in this study made up approximatelly 0.05% of total anaerobic bacteria present in the litter. A previous study found an average of 6.91 logs of anaerobic bacteria which is comparable to the 6.63 log overall average found in this study (Barker et al., 2010).

The recovery of aerobic bacteria over all six visits followed the same trend as the anaerobic bacteria with the first visits exhibiting a much lower recovery rate than the other four visits. It is probable that the decreased recovery of aerobic bacteria on the first visits is due to the same reasons seen for the anaerobic bacteria except for the availability of oxygen. In addition, house conditions prior to chick placement were not ideal due to decreased house temperatures and a lack of fresh fecal matter that would encourage bacterial growth. However, recovery of aerobic bacteria was much higher with an average of 8.19 logs (average 6.63 logs of anaerobic bacteria recovered). Visits 1B and 2B were both statistically significant by location within the houses and exhibited the same pattern. The two front quadrants of the houses had the highest levels of aerobic bacteria and were significantly different from the right rear quadrants of the

house. As already stated, this is most likely due to the increased activity at the front of the house while unloading chicks from the hatchery. A study by Barker et al. (2010) found that there was an average of 7.57 logs of aerobic bacteria in poultry litter, which is also very similar to the average recovery rate by this study of 8.19 logs. Another study found 9.0 logs of aerobic bacteria in poultry litter (Lu et al., 2002). The difference in aerobic bacteria numbers recovered in this study compared to previous studies could be explained by differences in farm location, flock health status, age of the litter, method of sample collection and culture methods.

There was no statistical significance for house type, ventilation type, brooding type, presence of wild birds, or mortality for any visit or farm over the entire project. High mortality was noted during several visits and while there was no statistical significance it is important to note that during these visits the cloacal swabs for the affected farms did not report a higher incidence of *Salmonella* or *Campylobacter.* The recovery of these two pathogens during the periods of higher mortality were actually overall much lower than for the visits and farms that exhibited normal mortality.

The *C. perfringens* PCR results from this study indicated that only 10.38% of the analyzed isolates had the *netB* gene and zero percent had the *tpeL* gene. Previous studies have found that NE derived isolates were positive for *netB* 4-70% of the time and between 0-9% were positive for *tpeL* (Keyburn et al., 2010; Martin and Smith, 2009; Bailey et al., 2015). However, it is important to note that these previous studies examined isolates obtained mostly from birds, some infected with NE while others were healthy. This study examined isolates taken exclusively from poultry litter. While the results of this study fell within the percent ranges found by other studies, our results were on the low end of each range. This is most likely due to several factors such as isolate collection location, sample source and that there was no NE on the farm.

As already mentioned, the previous studies examined isolates taken almost exclusively from birds, not poultry litter. Also, many isolates previously examined were taken from Europe and Australia where the gene was first identified. The study by Bailey et al. (2015) examined isolates collected over 15 years ago. Each of these factors could influence the presence of the gene due to the differing microbial populations present in different locations, sample type and year.

While the pathogen recovery results from this study were inconsistent with previous research, this can be explained partly by the local conditions at the time of sample collection, health status of the flocks, biosecurity and management practices performed, sample processing methods used and the type of farm samples were collected from. As stated previously, the goal of this study was to determine what effect that biosecurity and management practices have on the prevalence of the pathogens *Salmonella, C. perfringens* and *Campylobacter* in the poultry production environment. No changes were made to the biosecurity and management program as a result of our program recommendations so the lack of differences between the two sampling times come as no surprise. However, it is evident that both *Salmonella* and *C. perfringens* are consistently present in the poultry house environment which allows for introduction of these pathogens to future flocks if proper sanitation procedures are not performed consistently. The PCR results for *C. perfringens* isolates were consistent with previous findings and contribute to the growing body of evidence that suggests that *tpeL* is not as important of a virulent factor as previously thought and that while *netB* is an important virulent factor it is inconsistently present in *C. perfringens* isolates. Since there was no indication that the farms used for sample collection in this study were positive for NE, and were otherwise healthy, it is interesting that *netB* was found in 10% of the collected isolates. It is known that *C. perfringens* is not always the only microbe necessary to cause NE, cocci and conditions that stress the gastrointestinal tract are

often required as well. Given that cocci are ubiquitous in the poultry house environment, and the high prevalence of *C. perfringens* found in this study, it is evident that despite the presence of the two components needed to cause NE, there apperently was no disease. This could be partly due to the lack of gastrointestinal stress that occurs from certain feed stuffs, illnesses, and environmental triggers.

Future research in this area should be directed towards determining which specific practices influence the prevalence of pathogens on the poultry farm. In conjunction with this it is important to work with an integrator that is willing to make the recommended changes during the sampling period so that an accurate comparison could be made.

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Table 4.1. Characteristics for the farms that were sampled.

Farm Number	Ventilation Type	House Type	Brooding Arrangement	House Dimensions in feet (WxL)
1	Conventional	Curtain	Front End, Split*	40x400
2	Conventional	Curtain	Front End, Split*	40x400
3	Conventional	Solid Wall	Half House**	40x500
4	Conventional (2), Tunnel (2)	Curtain	Front End, Split*	40x400

*The front half of the house was sectioned off for brooding. Brooding fence was ran from the middle of the house to the front of the house along both interior water lines. This left an open walkway down the middle of the half of the house.
**The front half of the house was sectioned off for brooding, chicks were allowed full access to the entire portion of this house.

Table 4.2. Primer specifications used in multiplex PCR

Primer	Sequence	Target Gene	Product Length
netB5F*	CGCTTCACATAAAGGTTGGAAGGC	*netB*	
netBR*	TCCAGCACCAGCAGTTTTTCCT	netB	316
AKP80**	ATATAGAGTCAAGCAGTGGAG	*tpeL*	
AKP81**	GGAATACCACTTGATATACCTG	*tpeL*	466

*Previously published by Bailey et al, 2013
**Previously published by Keyburn et al, 2010 and Bailey et al, 2013

Table 4.3. Sample collection information for *Clostridium perfringens* isolates that tested positive for the toxin producing gene *netB* using PCR.

Number	Isolate ID	Isolate Collection Information		
		Visit	Farm	Location
1	1C-A1-B	1	1	1
2	1C-A2-D	1	1	2
3	1C-A3-A	1	1	3
4	1C-A4-C	1	1	4
5	1C-B1-A	1	1	1
6	1C-B2-A	1	1	2
7	1C-B3-A	1	1	3
8	1C-B4-A	1	1	4
9	1C-C1-B	1	1	1
10	1C-C2-A	1	1	2
11	1C-C3-A	1	1	3
12	1C-C4-A	1	1	4
13	1C-E4-A	1	2	4
14	1C-F3-C	1	2	3
15	1C-G1-A	1	2	1
16	1C-G3-D	1	2	3
17	1C-H1-A	1	2	1
18	1C-H3-D	1	2	3
19	1C-I3-D	1	3	3
20	1C-I4-A	1	3	4
21	1C-J1-C	1	3	1
22	1C-L4-C	1	3	4
23	1C-M1-C	1	4	1
24	1C-M4-A	1	4	4
25	1C-N1-C	1	4	1
26	1C-N2-A	1	4	2
27	1C-N3-C	1	4	3
28	1C-N4-A	1	4	4
29	1C-01-D	1	4	1
30	1C-03-D	1	4	3
31	1C-04-A	1	4	4
32	1C-P2-B	1	4	2
33	2C-E3-B	2	2	3
34	2C-F1-B	2	2	1
35	2C-F4-D	2	2	4
36	2C-G3-A	2	2	3
37	2C-H3-B	2	2	3
38	4C-G1-A	4	2	1

Table 4.4 BLAST search results for the sequenced PCR products. All isolates were positive for netB. The positive control used in this study was also sequenced. The primers used to obtain the PCR bands were netB5-F and netB5-R.

Isolate Number	Isolate ID	Most Significant Sequence	E-Value	Maximum Identity (%)
Positive Control	15C	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$1x10^{-136}$	98
1	1C-A1-B	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$2x10^{-124}$	95
5	1C-B1-A	*C. perfringens* strain CP4 plasmid pCP4netB pathogenicity locus 1 genomic sequence	$8x10^{-79}$	90
6	1C-B2-A	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$4x10^{-86}$	96
10	1C-C2-A	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$8x10^{-139}$	99
11	1C-C3-A	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$6x10^{-135}$	98

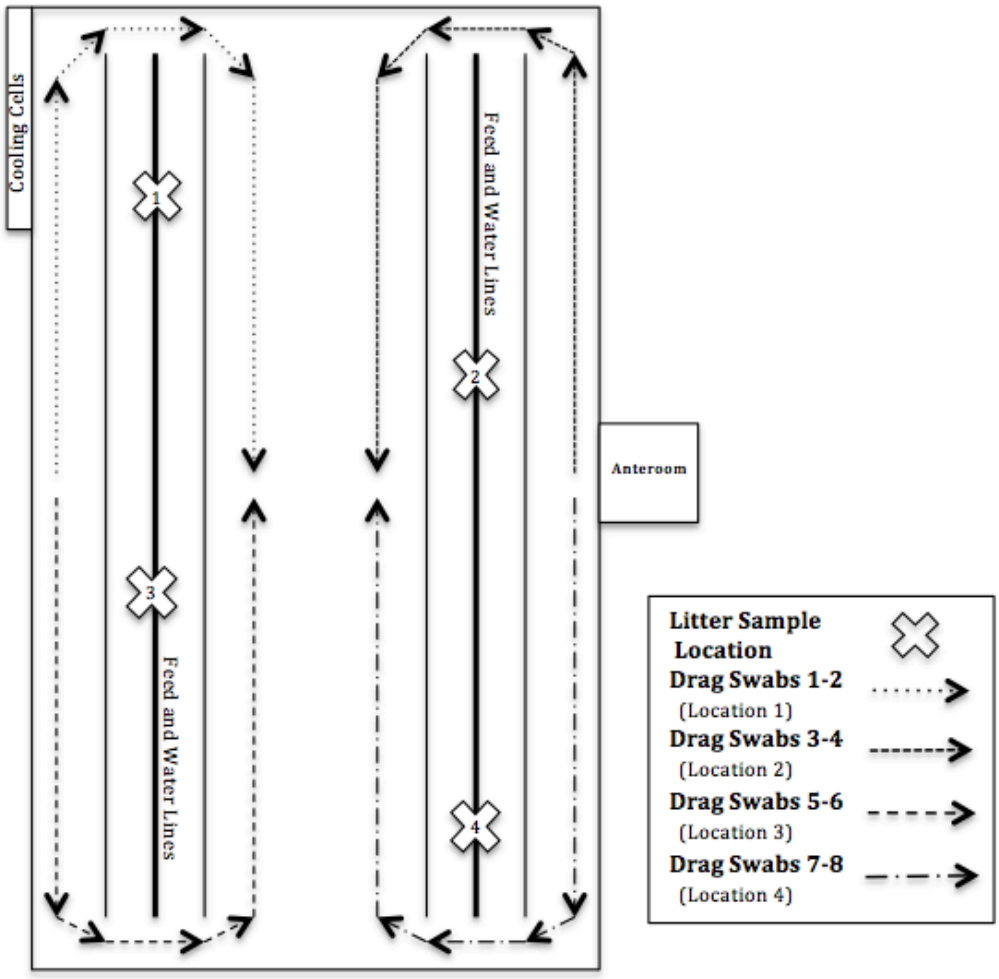

Figure 4.1. Sampling diagram for commercial poultry house. The back of the house is located by the cooling cells wheras the front of the house is at the opposite end.

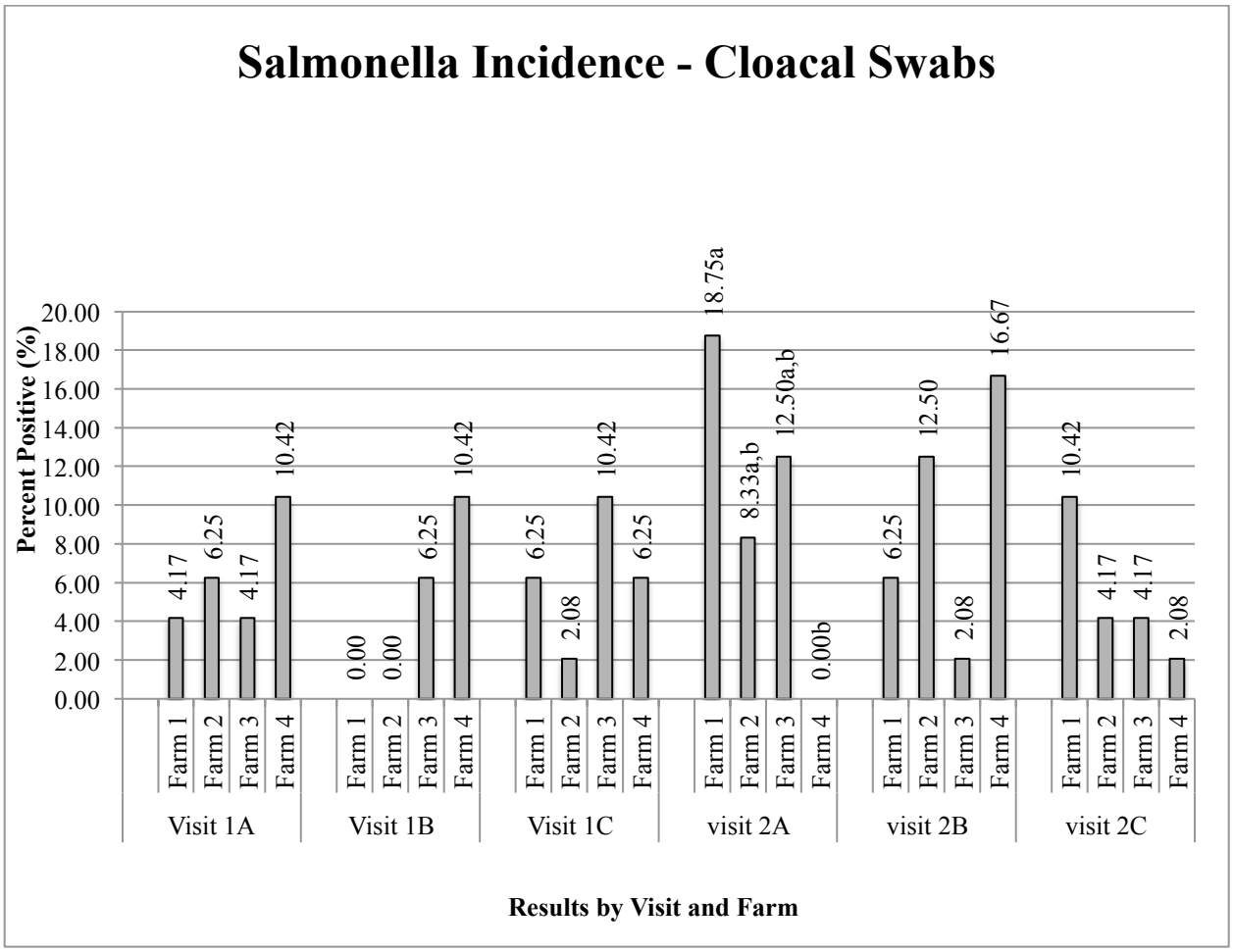

Figure 4.2. Percent positive samples for cloacal swabs enriched for *Salmonella* by visit and farm. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

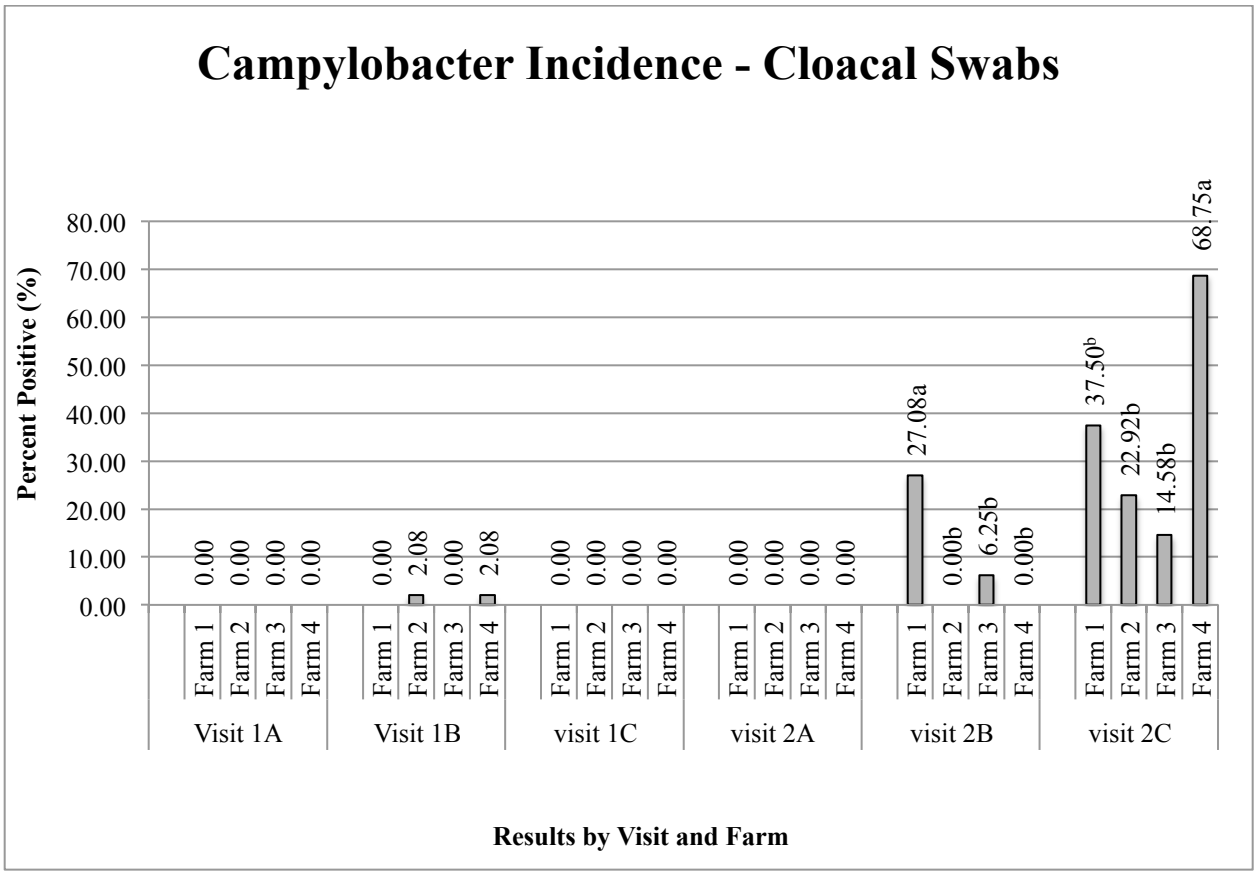

Figure 4.3. Percent positive samples for cloacal swabs enriched for *Campylobacter* by visit and farm. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

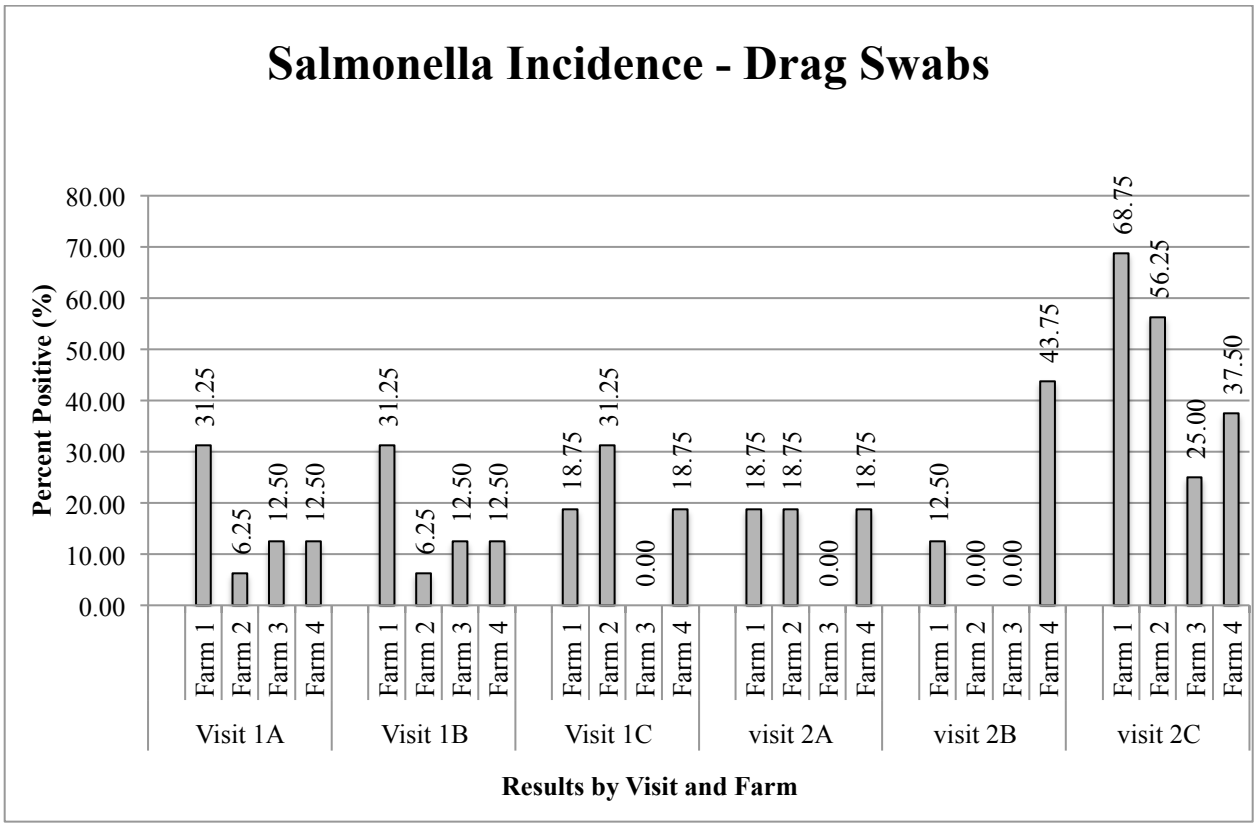

Figure 4.4. Percent positive samples for drag swabs enriched for *Salmonella* by visit and farm.

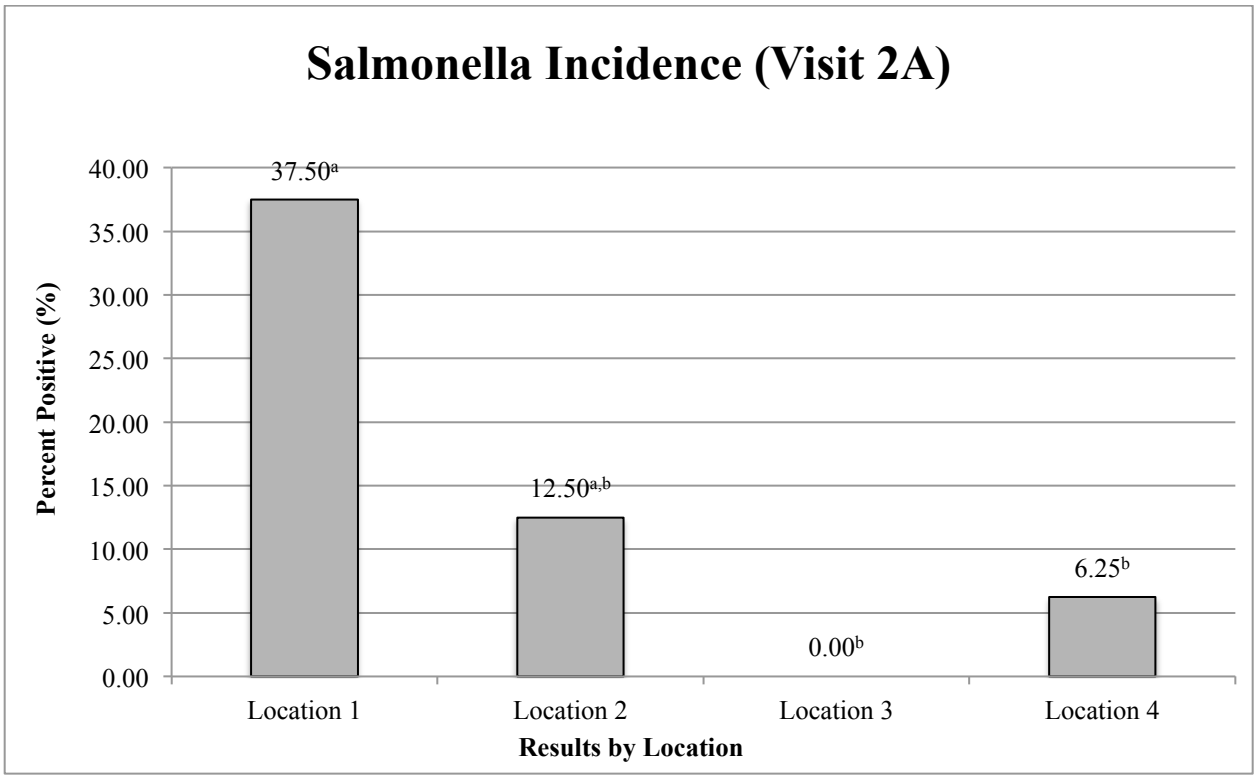

Figure 4.5. Percent positive results by house location for drag swabs enriched for *Salmonella* for visit 2A. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

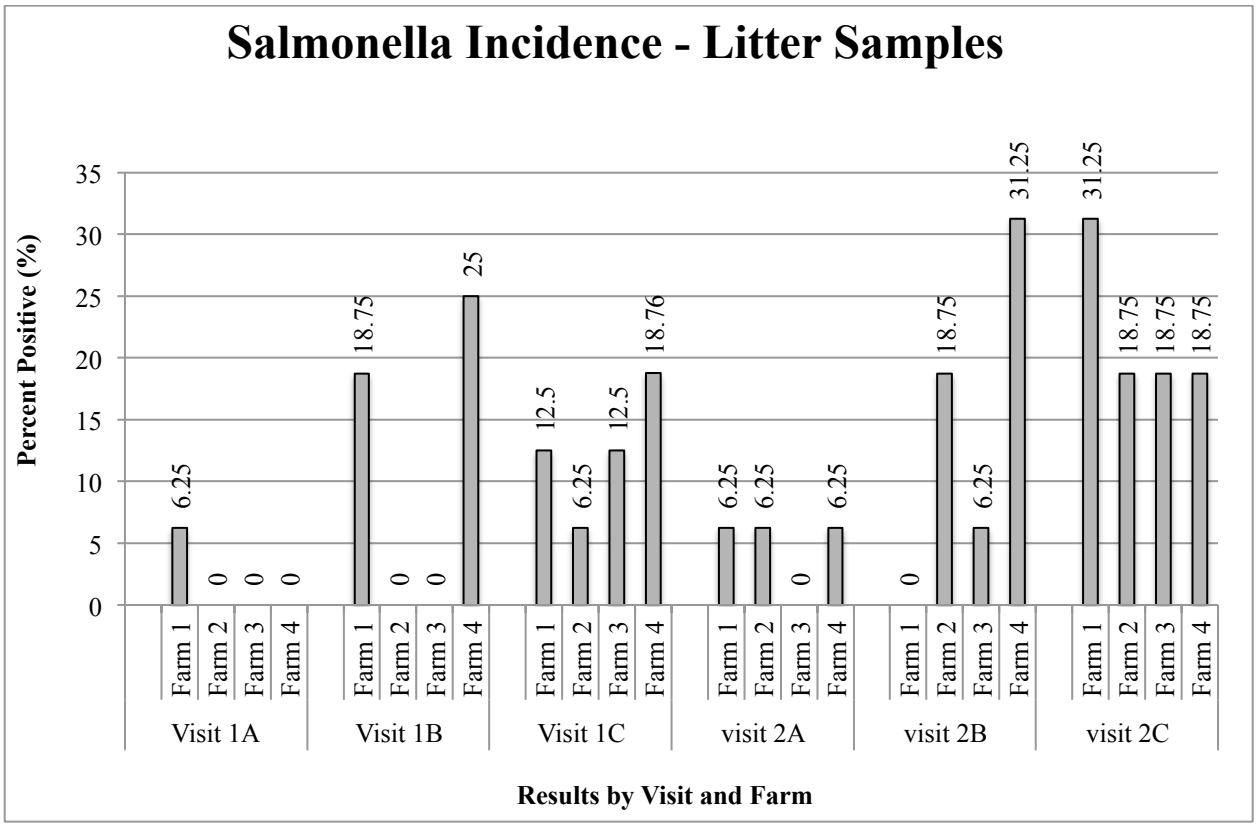

Figure 4.6. Percent positive samples for litter samples enriched for *Salmonella* by visit and farm.

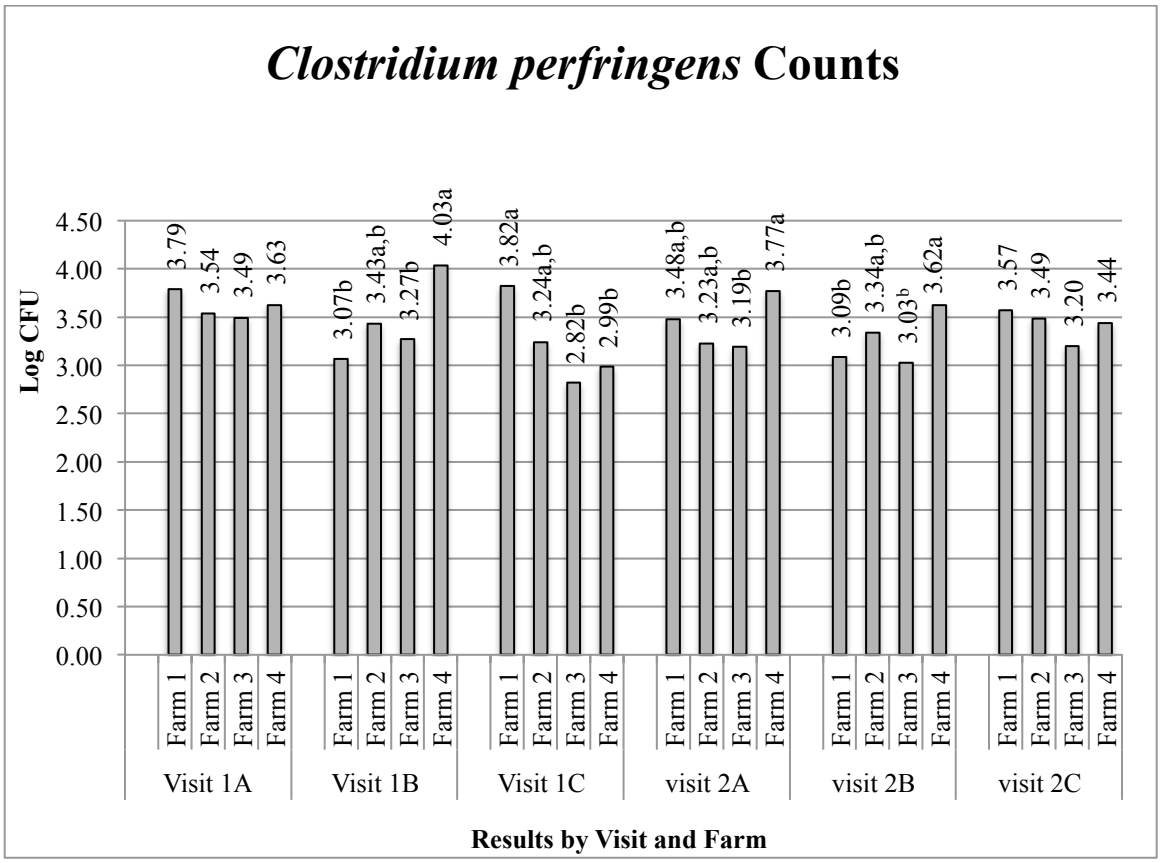

Figure 4.7. *Clostridium perfringens* bacterial counts recovered from litter samples by visit and farm. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

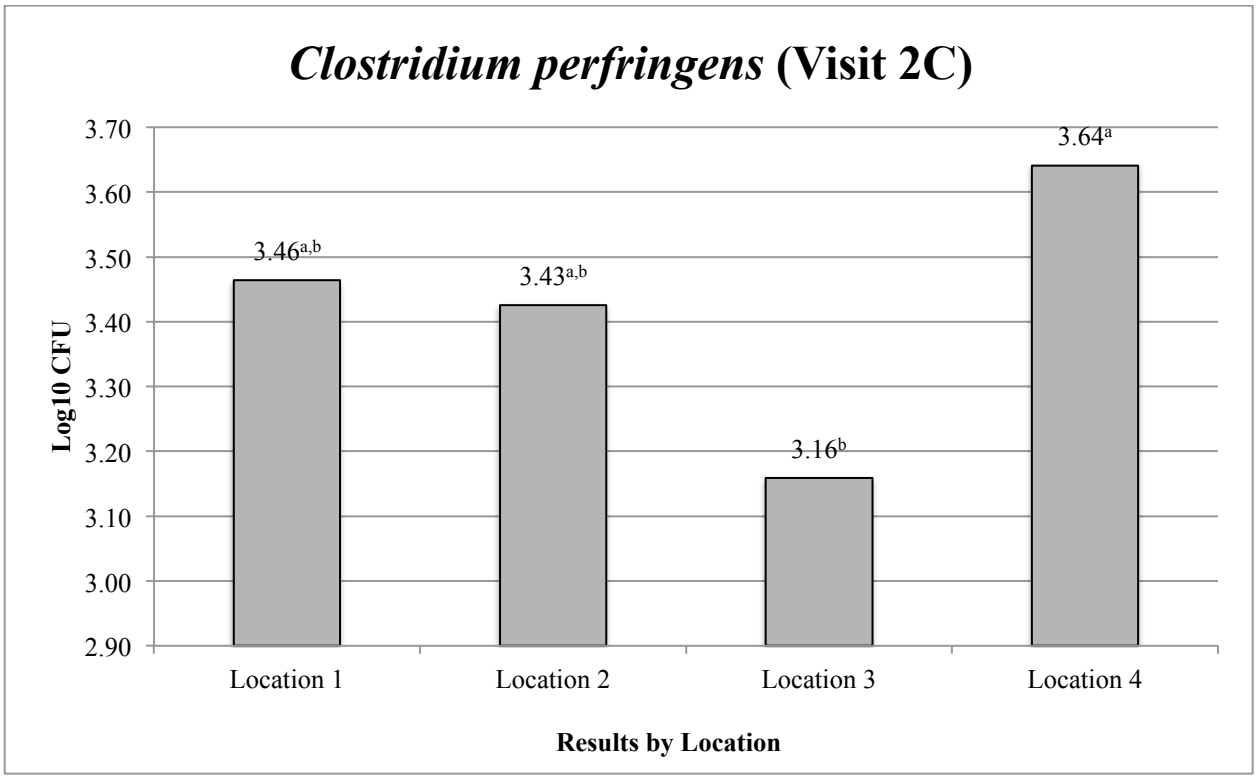

Figure 4.8. *Clostridium perfringens* log10 counts from litter samples by house location for visit 2C. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

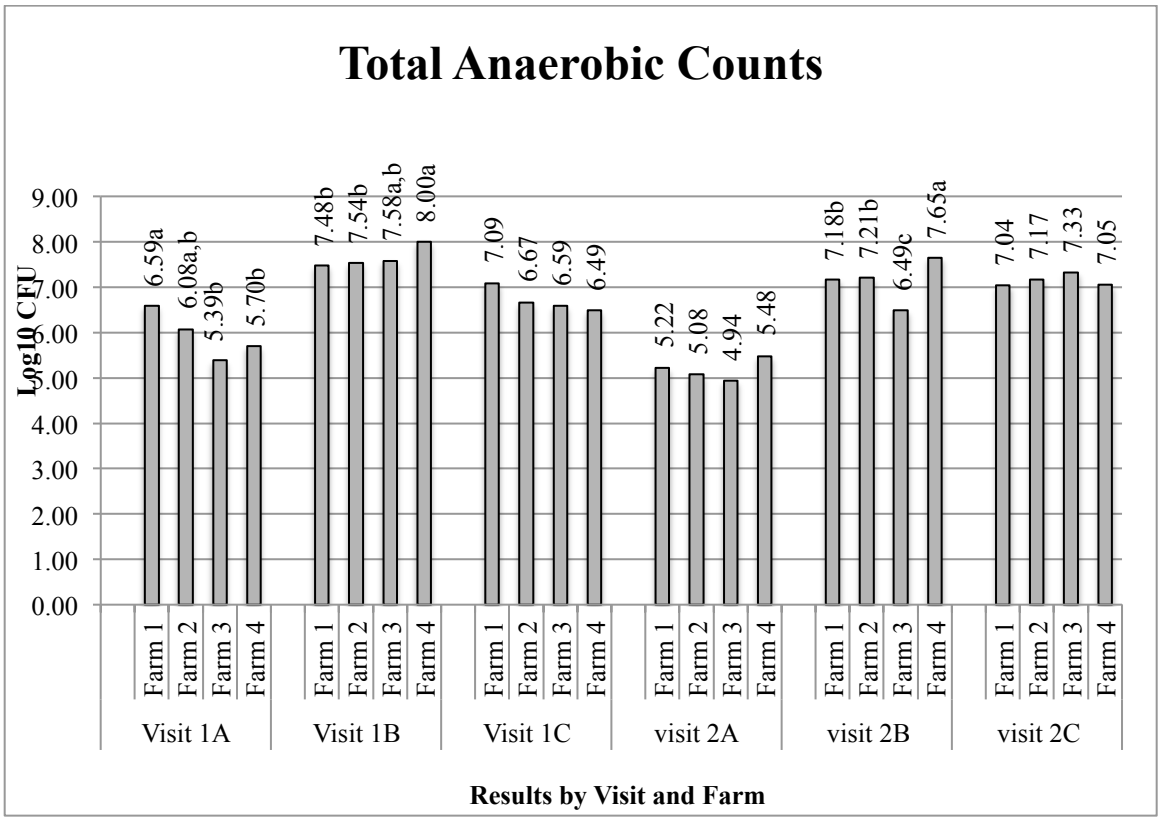

Figure 4.9. Total anaerobic bacteria recovered from litter samples by visit and farm. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

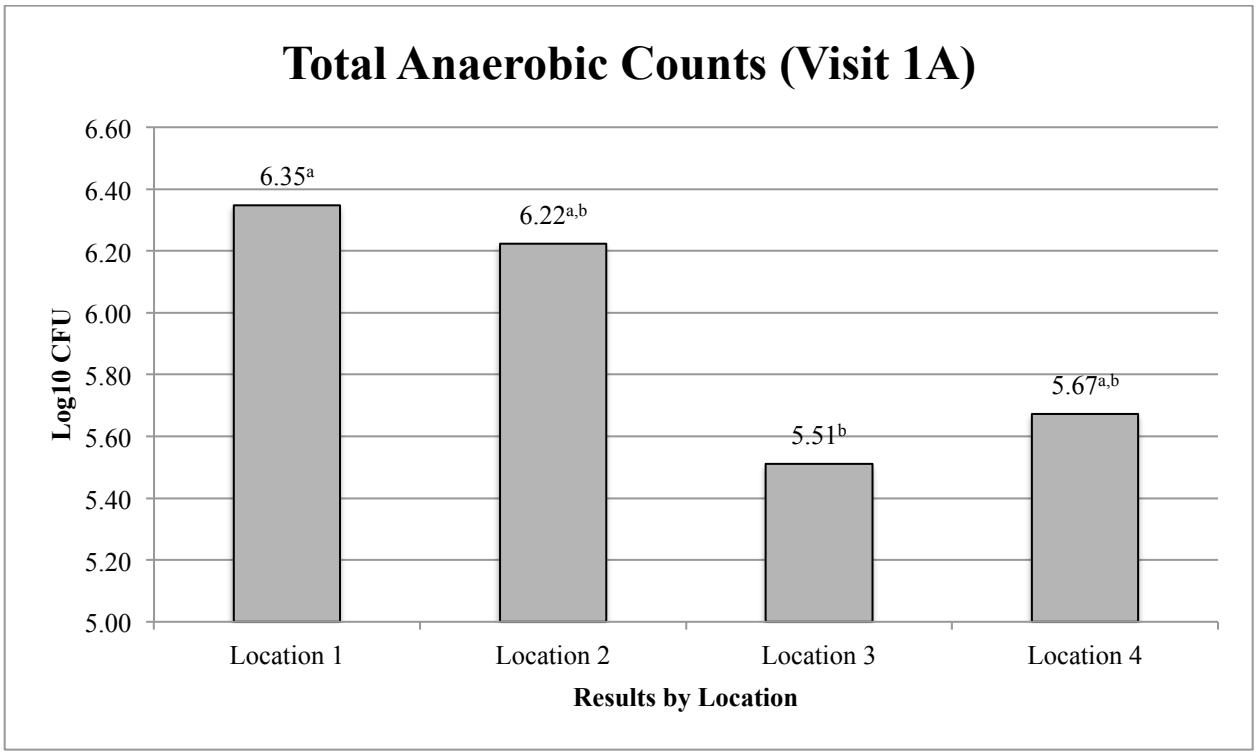

Figure 4.10. Anaerobic bacteria recovered from litter samples by house location for visit 1A. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

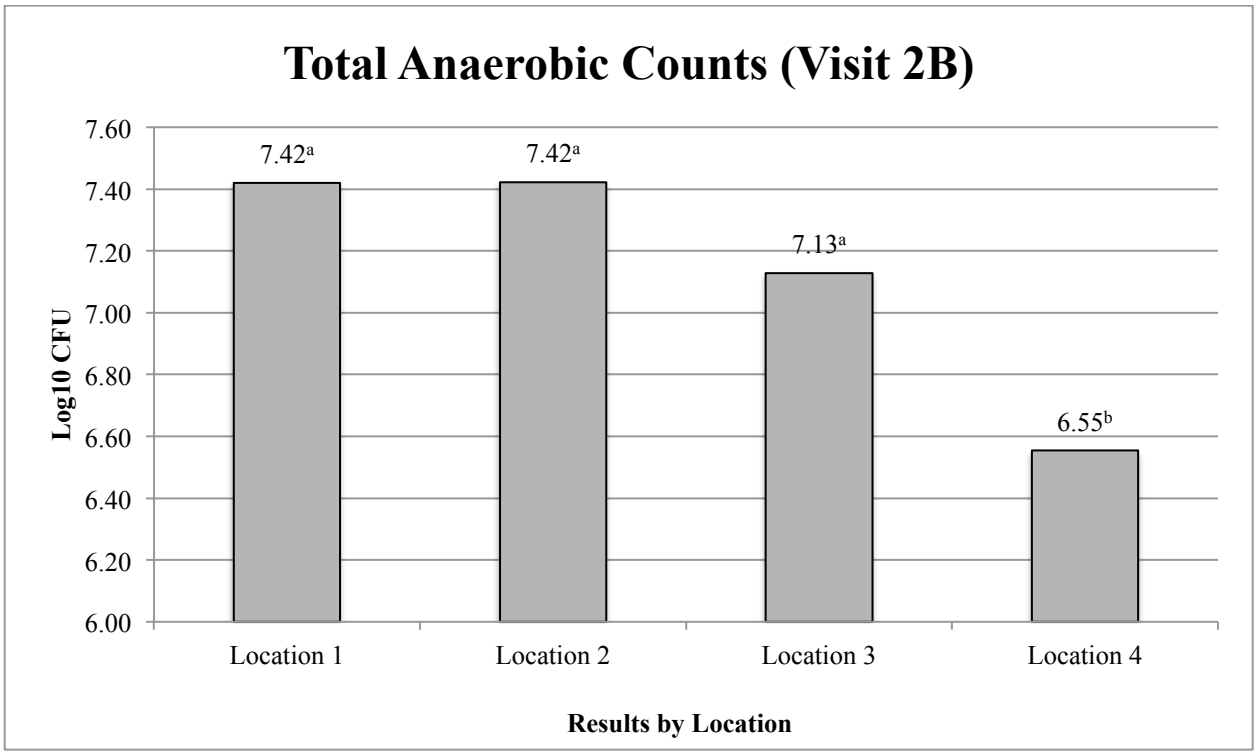

Figure 4.11. Anaerobic bacteria recovered from litter samples by house location for visit 2B. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

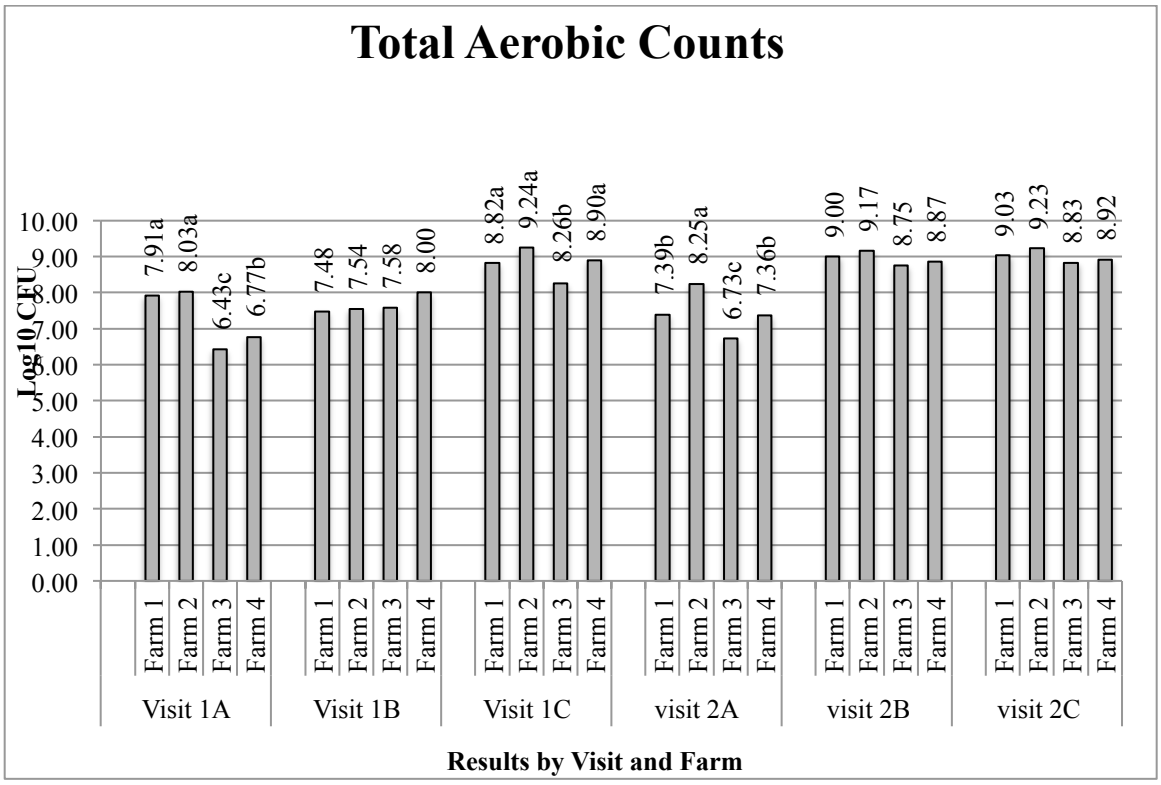

Figure 4.12. Log10 CFU counts of the total aerobic bacteria recovered from litter samples by visit and farm. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

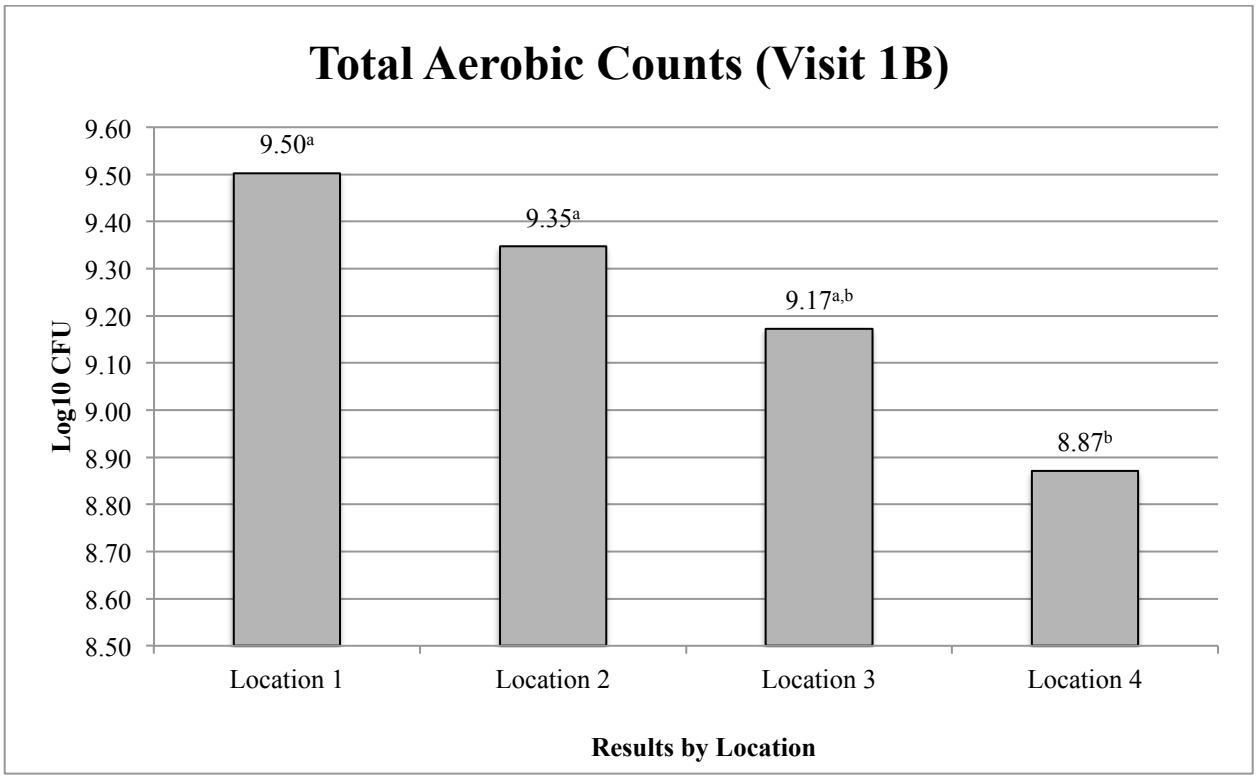

Figure 4.13. Aerobic bacteria recovered from litter samples by house location for visit 1B. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

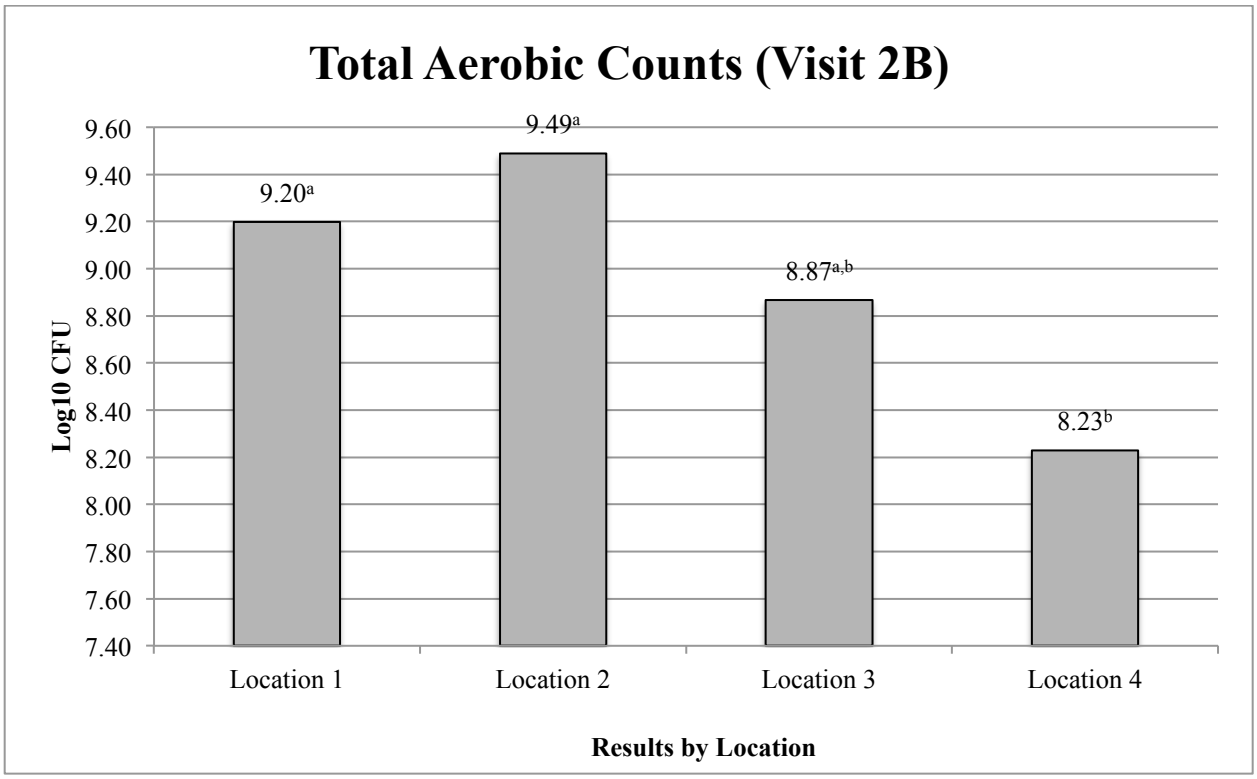

Figure 4.14. Aerobic bacteria recovered from litter samples by house location for visit 2B. Letter differences within a location constitutes a significant difference at $p \leq 0.05$.

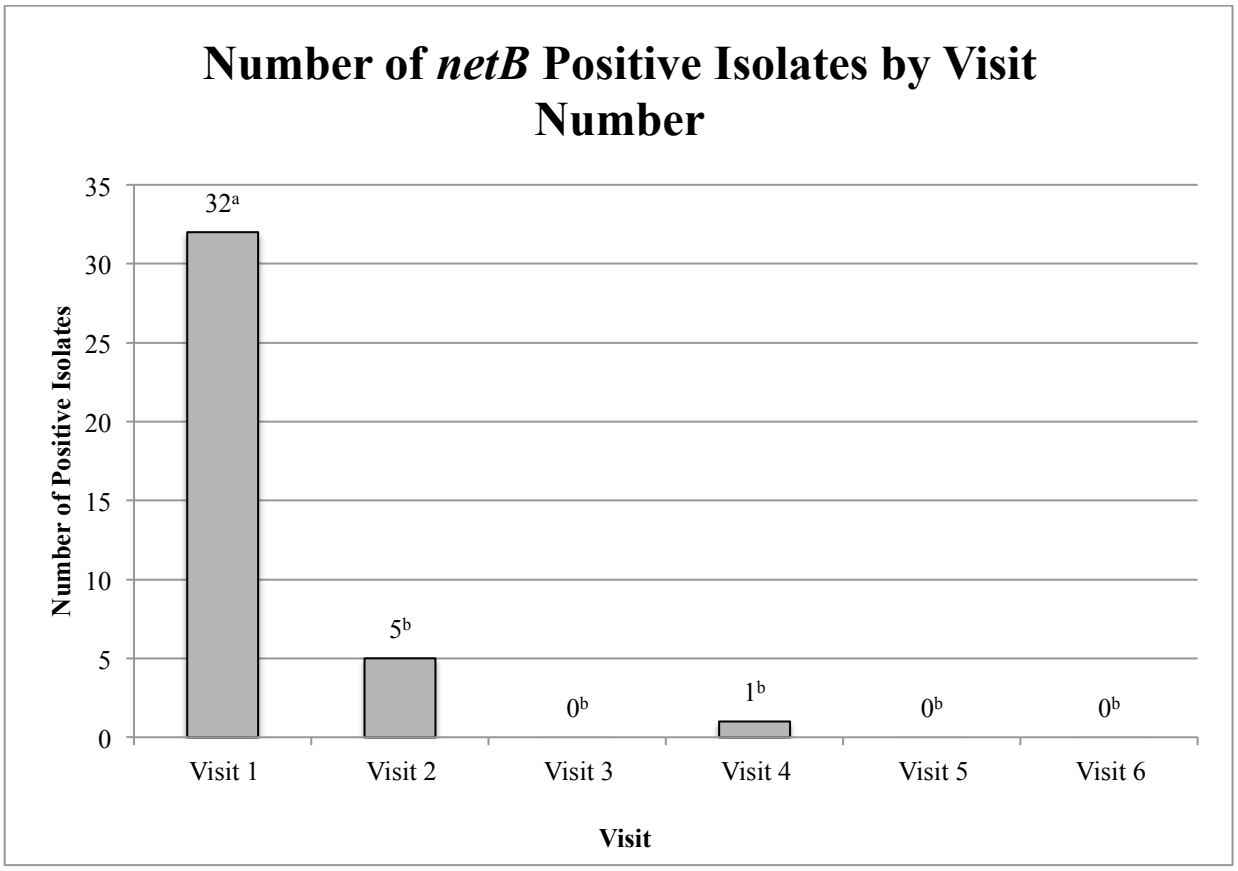

Figure 4.15. Number of *netB* positive *C. perfringens* isolates by visit number. Letter differences within a visit constitutes a significant difference at $p \leq 0.05$.

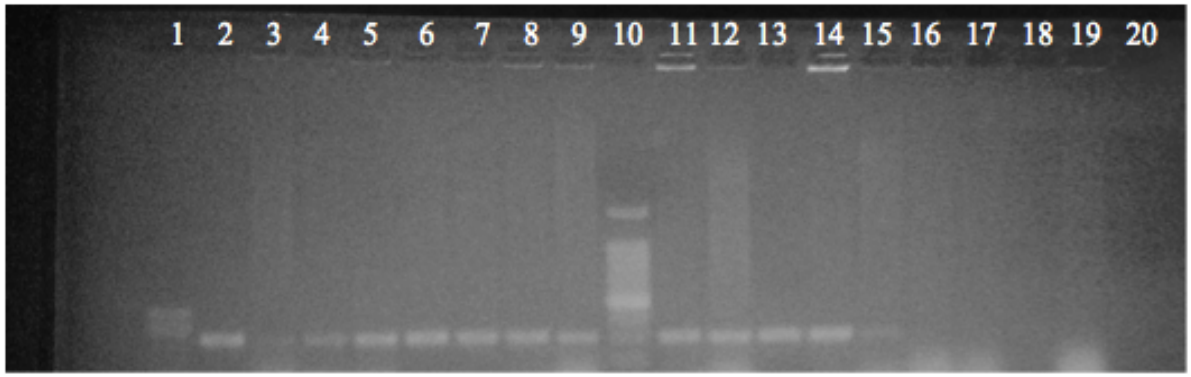

Figure 4.16. Photograph of gel electrophoresis for detection of *netB* and *tpeL* from *Clostridium perfringens* isolates. Lane 1 is the positive control from a *C. perfringens* isolate previously confirmed as positive for both *netB* (Lower band) and *tpeL* (Upper band). Lanes 2-9 contain isolates positive for the gene *netB*. Lane 10 is the Ladder. Lanes 11-15 contain isolates positive for the gene *netB*. Lanes 16-19 contain isolates negative for both genes. No isolates were positive for *tpeL*. Lane 20 is the negative control that did not contain any DNA.

V. Summary and General Conclusions

Salmonella, Campylobacter and Clostridium perfringens are three of the most common foodborne illness pathogens associated with poultry and as such are targets for control at all production points. The increase in vigilance is even more pressing due to recent changes to the regulation of these pathogens. This has caused the poultry industry to reevaluate the methods currently used to control these pathogens, specifically at pre-harvest points in production. The goal of this study was to determine what biosecurity and management practices directly influenced the bacterial prevalence on the commercial poultry farm.

The work presented in this thesis contributes to the current knowledge of how biosecurity and management practices affect the prevalence of various pathogens in the poultry production environment. Specifically, how the integrator influences the practices that the grower implements. It is evident from our results that despite our recommendations on biosecurity and management practice improvements, the integrator and/or the poultry farmers failed to implement these changes resulting in few statistically significant differences between both surveys and sampling periods. Our results are similar to those found by a previous study that found that unless disease was present, poultry farmers were not likely to strictly follow biosecurity and management practices as outlined by the integrator due to the increased cost involved (Dorea et al., 2010). It is the responsibility of the integrator to properly educate growers on the importance of these practices and enforce biosecurity and management practices that provide adequate coverage to prevent the occurrence and spread of disease. It is also the responsibility of the farmer to properly implement the practices they learned about, and maintain their use on a daily basis. Because of this, it is essential that future research and regulatory efforts in this area be focused on both the integrator involvement, and the poultry farmers.

Despite the overall lack of statistical significance for the bacterial samplings, there are three points worth mentioning. First, all three pathogens of interest (*Salmonella, Campylobacter, and C. perfringens*) were found to be readily available in the poultry house environment indicating that it can act as a reservoir of infection for current and future flocks. Second, *Salmonella* was found in up to 31.25% of the samples on the first visits. Both litter samples and cloacal swabs were positive for *Salmonella,* which implies that the chicks were contaminated either at the hatchery or from the breeder flock. This means that the current management and biosecurity practices performed on these farms are either not effectively reducing pathogen

levels between flocks or that the contaminated chicks are nullifying these practices by introducing *Salmonella* back into the environment. Third, both *Salmonella* and *Campylobacter* were present at high levels in the samples taken on the last visits for each sampling period. Since these flocks were due to be sent to the processing facility a few days after our last visit, it is safe to say that these flocks were contaminated with these pathogens when they were processed making them a source of contamination within the processing plant.

C. perfringens was also found during all visits over both sampling periods. However this is not unexpected as this organism is commonly present in both the environment and natural gut micro flora. The issue with this pathogen is that some strains are pathogenic to both humans and avian species and recent research has linked several toxin producing genes (*netB* and *tpeL)* to strains that cause NE in poultry. Previously it was thought that both of these genes were major virulent factors that could potentially be used to both track disease and as targets for disease prevention strategies. However, recent research has indicated that both genes are not as prevalent as previously expected, especially the gene *tpeL.* To date, the prevalence of these genes in healthy, NE free flocks is generally unknown. This study contributes to this knowledge base, especially for the presence of *netB* and indicates that this gene is naturally present in *C. perfringens* isolated from poultry houses.

The results of this study were unable to directly identify which specific biosecurity and management practices influenced bacterial prevalence on the commercial poultry farm. Despite this fact, valuable information was collected regarding common on-farm biosecurity and management practices, their compliance, and the microbial population of various sample types taken from a poultry house environment. While the integrator and farmers failed to implement the recommended changes and continued to carry out their normal biosecurity and management programs, it is evident that overall their compliance with these programs is fairly consistent. Few differences were noted between survey and sampling periods because of this lack of change in program implementation. Important to note is that for the duration of this study, widespread disease was not present in this region. The lack of a complete and consistent implemented biosecurity program is most likely due to a lack of disease. During times of apparent health, biosecurity and management practices do not seem as important to the growers who are responsible for the total cost of these practices and as such often fail to properly maintain these programs. Shortly after the end of this study, AI broke out in the Midwest, spiking interest in

biosecurity and management practices which most likely would result in increased compliance should the same farms be studied again.

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