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Salmonella Source and Rate of Colonization in Two Newly Constructed Commercial Broiler Houses and the Effect of Used Litter Inoculation in a New House

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By

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The prevalence of *Salmonella* within poultry environments and on poultry products has been well documented. However, there has not been a study documenting the effect of utilizing used litter in newly constructed commercial broiler houses on *Salmonella* status or on the rate and source of *Salmonella* contamination within new houses. Objectives of this study are to 1) determine environmental source and rate of *Salmonella* spp. colonization in two newly constructed broiler houses 2) to evaluate the effect of mixing used broiler litter with clean litter in a new broiler house.

Results of this study suggest that *Salmonella* contamination of the poultry house environment occurred within the first 2-4 weeks of bird placement and that the source of contamination may have been the chicks themselves. Litter inoculation may be beneficial in reducing *Salmonella* levels within the first flock if it is known that the chicks are already contaminated with *Salmonella* spp.

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CHAPTER I

LITERATURE REVIEW

Introduction

It has been well documented that *Salmonella* spp. are prevalent within poultry environments and that poultry house contamination is associated with end product contamination. This review of the literature is intended to give a general overview of the modern United States poultry industry and issues associated with the industry, to give a general overview of *Salmonella*, and to outline previously conducted studies on *Salmonella* within poultry environments.

Economics

The United States is the largest poultry producer in the world, producing approximately 43 billion pounds of poultry meat each year (USDA-ERS, 2009). The U.S. is also the second largest producer of eggs and second largest exporter of poultry behind Brazil (USDA-ERS, 2009). In 2006 the average American citizen consumed 86 pounds of chicken per year as compared to 28 pounds in 1960; this is a three-fold increase resulting from increased income, inexpensive prices of poultry in comparison to alternative meats, and movement of consumer preferences (MacDonald, 2008). Americans eat more poultry than either beef or pork, but still less than all combined red

meat at this time (USDA-ERS, 2009). Broiler meat accounts for about 80% of the poultry meat market; these producers are predominantly located throughout the southeastern United States with the top five broiler producing states being Georgia, Alabama, Arkansas, Mississippi, and North Carolina (USDA-ERS, 2009; US Poultry and Egg Assoc, 2012.). Poultry production in Mississippi is concentrated in the central part of the state with Leake, Neshoba, Scott, Newton, Franklin, Smith, Simpson, Jones, and Wayne counties being the top broiler producing counties in the state, each producing more than 150 million pounds annually (Kidd et al, 2007). Poultry was Mississippi's top agricultural commodity for 2011 with an estimated $2.4 billion value with forestry and soybeans following (Collins-Smith, 2011). Mississippi produces a broiler surplus of approximately 88% annually giving the state a healthy export market, within the US and internationally (MSU Extension Service, 2010).

Structure of Modern U.S. Poultry Industry

The modern U.S. poultry industry is one of the agricultural commodities that have been vertically integrated to encompass all aspects of the farm to table production within one company; other agricultural commodities that have adopted this system include the catfish and pork industries. This organizational system began during the 1950's when poultry companies began trending towards purchasing the other segments of the industry to facilitate decreased costs, improved record keeping, implementation of industry and scientific updates, and to produce a single profit source (MSU Extension Service, 2010). The companies usually own the hatcheries, feed mills, and processing plants. Broiler

production is usually organized with integrator companies contracting broiler grow-out through an independent farmer who supplies the land, poultry houses, equipment, labor and utilities (all or part). The integrated poultry company in turn provides the chicks, feed, veterinary services, and sometimes a portion of the utility costs (MacDonald, 2008). They also provide transport from the farm to the processing facility and usually provide the labor required for catching and loading the birds on to the transport trucks at the end of the grow-out cycle.

Broiler grow-out operations contain on average three to four broiler houses, while each house can hold approximately 23,000 birds. Older farms usually have fewer houses, while newer farms are trending towards larger operations (MacDonald, 2008). Typically the birds are grown for approximately six weeks, but can vary depending on the market demand for certain size birds. Approximately two to three weeks between flocks gives the farmer a chance to ready the houses for the next flock. Tasks to be completed prior to arrival of the new flock include: de-caking (removing the top layer of cake from the litter), leveling, greasing augers, blowing dust from the walls, ceilings, and fans; applying litter treatment to reduce ammonia during the winter and spraying for litter beetles. Given the six week flock cycle and the two to three weeks it takes to receive new chicks, a typical farmer can produce 5-6 flocks per year.

Broiler houses within the United States are constructed to be approximately 400-600 feet long and 40-50 feet wide with 8 foot high suspended ceilings supported by trusses to negate the need for support columns within the house (Fairchild, 2005). Houses usually are situated east to west to reduce the amount of solar heat produced by the sun shining on the side walls. Newer houses are well insulated and built with dropped

ceilings in order to decrease costs associated with heating and to improve ventilation (Fairchild, 2005). Newer houses are also built with solid side walls as opposed to the old design with open sides and curtains. The insulated solid side walls help facilitate improved tunnel ventilation for bird cooling as well as allowing for more controlled lighting and temperature (Fairchild, 2005). Ventilation is achieved by using fans to pull fresh air down the length of the house (i.e. tunnel ventilation); this helps maintain air quality and comfortable temperature and humidity. During hot weather, cooling is optimized by pulling the air through water soaked cool pads on the outside of the house, taking advantage of evaporative cooling to lower the air temperature by approximately ten degrees (Fairchild, 2005). During cool weather the houses are most often heated by propane or natural gas heaters throughout the house; to keep from wasting trapped heated air that rises to the ceiling, circulation fans are sometimes used to push the warmer air back down to floor level (Fairchild, 2005). Air is exhausted out of the ceiling and fresh air is pulled into the house through inlets that are high on the walls or in the ceiling; these inlets are placed high in the house to allow the air to warm before reaching floor level (Fairchild, 2005). The floor is comprised of packed dirt with some type of bedding applied, usually wood shavings, rice hulls, peanut shells, or the like. This bedding becomes the "litter" which is comprised primarily of bedding, excreta, and wasted feed.

Confined Animal Feeding Operation

In 1972 Congress enacted the Federal Water Pollution Control Act, more commonly known as the Clean Water Act (CWA). The objective of this act is to "restore and maintain the chemical, physical, and biological integrity of the Nation's waters"

(CWA section 101(a)). Covered under this act is the authority of the Environmental Protection Agency to regulate release of any pollutants from the point sources to water (CWA section 402). Specifically included within the Clean Water Act are Confined Animal Feeding Operations (CAFO) as point sources, although a formal definition of CAFO was not provided until 1976. In order to be designated as a CAFO the operation must first meet the requirements to be designated as an Animal Feeding Operation (AFO). Under 40 CFR 122.23(b)(1) an AFO is defined as " a lot or facility where animals have been, are or will be stabled or confined and fed or maintained for a total of 45 days or more in any 12-month period and crops, vegetation, forage growth, or post-harvest residues are not sustained in the normal growing season over any portion of the lot or facility"(Protection of Environment, 2010). A CAFO is an Animal Feeding Operation that meets federal regulatory definitions of large, medium, or small CAFOs based on confined animal numbers outlined in 40 CFR 122.23(b)(4),(6) or (9) (Protection of Environment, 2010). An AFO can also be designated as a CAFO by the National Pollutant Discharge Elimination System (NPDES) permitting authority or by the EPA as outlined in 40 CFR 122.23(c) (Protection of Environment, 2010). Broilers fall under the animal sector category "chickens other than laying hens (other than a liquid manure handling system)" and are designated as a large CAFO if the threshold number of 125,000 birds or more per farm is met. A medium broiler CAFO is one that reaches the threshold of 37,500-124, 999 birds, and a small CAFO is one that confines less than 37,500 birds. The designation of CAFO is important in the poultry industry since most poultry production farms fall under this definition and are subject to regulations and inspections by the NPDES and EPA. All of these large broiler grower operations must

obtain a permit from the state permitting authority or from the EPA. There are regulations under 40 CFR pt 122 regarding handling and storage of litter and feed, land application of litter, transfer of litter off of the farm, and record keeping (VanDevender, 2003; Protection of Environment, 2010). These regulations require that if manure is stored outside of production facilities that it be stored under a permanent stacking shed that is protected from the weather or covered by a tarp if being stored temporarily (VanDevender, 2003; Protection of Environment, 2010). Regulations regarding land application of poultry litter as fertilizer designate that all fields to which litter is to be applied implement setbacks, buffers, or other conservation practices that protect surface water (Henry, 2003; Protection of Environment, 2010). Litter may not be applied any closer than 100 ft to any down gradient surface waters, sinkholes, agricultural well heads, open tile intake structures, or other channels to surface waters; an alternative to the 100 ft setback is a 35 ft vegetative buffer that may be used instead (Henry, 2003; Protection of Environment, 2010). Large CAFOs are required to keep records on removal of litter from the farm regarding who the litter was transferred to with their contact information, the date, and the amount of litter transferred. When litter is removed it is the farmer's responsibility to ensure that an environmentally friendly process of utilizing this product is followed. This is commonly done by land applying the litter as fertilizer, although this method has some limitations as well to protect surface waters from excess phosphorous accumulation, which upsets the ecosystem balance and can cause algal blooms and fish kills (Wiederholt and Mathews, 2012). Other litter concerns include nitrate reaching the ground water supply which can cause methemoglobinemia and spontaneous abortion in

humans; and exposure of humans to pathogens association with animal waste (Bowman et al, 2000).

Food Safety and Poultry

It is estimated that each year in the United States 9.4 million foodborne illnesses occur, with 3.6 million of these illnesses associated with bacteria (Scallan et al, 2011). Non typhoidal *Salmonella* spp. was the second leading cause of foodborne illness within the United States, ranking only behind Norovirus (Scallan et al, 2011). Although it ranked behind Norovirus with regard to illnesses, non typhoidal *Salmonella* spp. was the leading cause of hospitalizations caused by foodborne illness annually with an estimated 23,128 hospitalizations and was also the leading cause of mortality in the U.S. due to foodborne illness with an estimated 452 annual deaths (Scallan et al, 2011). It is estimated that *Salmonella* infections cost the United States $365 million dollars annually in direct medical costs, and with all factors considered (e.g. lost wages, loss of life, hospitalization, outpatient costs), costs the U.S. approximately $2.8 billion dollars annually (Adhikari, 2004 and Gilliss, 2010).

Healthy People 2010 national health objectives laid out by the U.S. Department of Health and Human Services and by the Office of Disease Prevention and Health Promotion set incidence goals for key foodborne pathogens: *Campylobacter*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* sp., all of which are monitored by FoodNet (U.S. Dept HHS). Monitoring began during 1996-1998, and the 2010 trend report showed a significant decrease in *E. coli* O157:H7 incidence that reached the 2010 Healthy People goal, while the incidence of *Campylobacter* and *Listeria* showed no

significant change and did not reach outlined incidence goals (Gilliss et al, 2011). The incidence for *Salmonella* infections in 1996-1998 compared to 2010 showed no significant difference; however there was a significant increase (10%) in infections between 2006-2008 and 2010 (Gilliss et al, 2011). *Salmonella* showed the least amount of progress towards reaching outlined goals and even increased in incidence in the most recent years, making it an infection to pay closer attention to over the coming years as we move toward the Healthy People 2020 incidence goals.

Salmonella is commonly found in the intestines of healthy animals, which can contaminate the environment to the extent that agricultural crops and produce are possibly contaminated, and contamination can occur during slaughter and processing of food animals. Different serotypes of *Salmonella* are associated with different food products, but as of 2010 the *Salmonella* serotype that caused the most human infections in the United States was *Salmonella* serotype Enteritidis followed by serotypes Newport and Typhimurium (Gilliss et al, 2011). A common source of *Salmonella* serotype Enteritidis is eggs and poultry; other *Salmonella* serotypes are commonly identified in poultry as well, making poultry an important source of human *Salmonella* infection (Altekruse et al, 2006).

Organism Description (*Salmonella*)

Salmonella are gram negative, rod shaped bacilli belonging to the family *Enterobacteriaceae*. They are facultative anaerobes, and most are motile via peritrichous flagella. Some strains are non-flagellated, while others have dysfunctional flagella. *Salmonella* are able to utilize a wide range of organic substrates and are also able to

metabolize nutrients by respiratory and fermentative pathways (Montville and Matthews, 2005). Their optimal growth temperature is 37°C; however some strains may grow at temperatures as high as 54°C, while others may grow at 2°-4°C.

Nomenclature: Kauffmann-White Scheme

To facilitate international congruency, the Centers for Disease Control and Prevention adopted the Kauffmann-White Scheme of *Salmonella* serotype designation in 2002 (Bishop et al, 2011). This nomenclature scheme is maintained by the World Health Organization Collaborating Center for Reference and Research on *Salmonella* at the Institut Pasteur in Paris, France and is utilized by most public health officials and laboratories throughout the world (Grimont and Weill, 2007; Bishop et al, 2011). Although this scheme is accepted by many, there is still considerable debate on name changes; because of differing names reported for the same organism, Brenner et al (2000) suggests that multiple versions of the serotype name be listed as key words in manuscripts to ensure compatibility with literature searches.

The genus *Salmonella* is comprised of two species: *Salmonella enteritica* and *Salmonella bongori*. The species *Salmonella enterica* is further subdivided into six subspecies which are appointed taxonomic names and abbreviated by Roman numerals, which are utilized in designation by formula (Grimont and Weill, 2007; Bishop et al, 2011). *Salmonella bongori* was previously thought to be a subspecies of *Salmonella enterica* and was given the abbreviation V for formula purposes which it still carries. The table below from Bishop et al (2011) displays the six subspecies of *Salmonella enterica* with the corresponding taxonomic names and abbreviations.

Table 1.1
The six subspecies of *Salmonella enterica* with corresponding taxonomic names and abbreviations (Bishop et al, 2011)

Salmonella enterica subspecies	
I	*Salmonella enterica* subsp. *enterica*
II	*Salmonella enterica* subsp. *salamae*
IIIa	*Salmonella enterica* subsp. *arizonae*
IIIb	*Salmonella enterica* subsp. *diarizonae*
IV	*Salmonella enterica* subsp. *houtenae*
VI	*Salmonella enterica* subsp. *indica*

Serotyping establishes further breakdown past the subspecies level. These serotypes are broken down based on the immunoreactivity of two cell surface antigens, O and H. The O antigen is a polysaccharide typically composed of four to six sugars that makes up a component of the cell surface lipopolysaccharide. O antigens are broken down into two groups: O group antigens and ancillary O antigens. The O group antigens are associated with the core sugar structure, while the ancillary O antigens are further carbohydrates that are added to the core O antigen configuration (Bishop et al, 2011). The O antigen is designated by a number for formula purposes, but the previous method designated O antigens by a letter, which is still sometimes used. The H antigen is the filamentous segment of the bacterial flagellum, which is made up of protein subunits called flagellin (Grimont and Weill, 2007; Bishop et al, 2011). *Salmonella* has the ability to express two different flagellin antigens, which are identified as Phase 1 and Phase 2

antigens; generally only one of these antigens is expressed at one time (Bishop et al, 2011).Currently there are more than 2,500 serotypes with additional serotype recognition regularly (Bishop et al, 2011). Pulsed Field Gel Electrophoresis (PFGE) pattern characterization allows for further subtyping that is useful for identifying outbreaks associated with particular subtypes.

When identifying serotypes of *Salmonella*, the genus and species are first identified followed by biochemical testing to differentiate among subspecies. Agglutination assays are utilized to identify specific O and H antigens by using antisera that react with related antigens. Once the *Salmonella* isolate is identified down to the serotype it can be reported as a formula following the Kauffmann-White Scheme; the format for identifying serotype formula is as follows: Subspecies, space, O antigens, colon, Phase 1H antigen, colon, Phase 2 H antigen (Grimont and Weill, 2007; Bishop et al, 2011). For example, the antigenic formula for *Salmonella enterica* serotype Typhimurium is I 4,5,12:i:1,2; however, under the Kauffmann-White Scheme it is allowable to use the original names for subspecies I; this outline provides the basis for *Salmonella* designation, and further details concerning specifics on formula derivation can be found in the 9th edition of the Antigenic Formulae Of The *Salmonella* Serovars (Grimont and Weill, 2007).

Pre-harvest Food Safety/Litter

Multiple studies have been performed to evaluate the microbial population present within poultry litter, especially in regards to *Salmonella, Campylobacter,* and other pathogenic bacterial populations (Lu et al, 2003; Terzich et al, 2000; Altekruse, et al,

2006; Arsenault et al, 2007; Wedderkopp et al, 2001; Santos et al, 2005; Kelley et al, 1998). Most studies are focused simply on *Salmonella* or *Salmonella* and *Campylobacter* prevalence since both are the number 1 and 2 bacterial causes of foodborne infection resulting in hospitalization, respectively (Wedderkopp et al, 2001; Santos et al, 2005; Altekruse et al, 2006; Arsenault et al, 2007; Scallan et al, 2011). Most of these studies were conducted using traditional culture methods, although Lu et al (2003) evaluated the litter population using 16S ribosomal RNA sequencing and polymerase chain reaction (PCR) screening for pathogens. Pathogens identified in these studies included: *Staphylococcus*, *Clostridium perfringens*, *Salmonella*, *Campylobacter*, *Escherichia coli*, *Bordetella* spp., *Streptococcus*, *Enterococcus, Pseudomonas, Yersinia,* and *Aeromonas* (Kelley et al, 1998; Terzich et al, 2000; Wedderkopp et al, 2001; Lu et al, 2003; Santos et al, 2005; Altekruse, et al, 2006; Arsenault et al, 2007). The prevalence of *Salmonella* within poultry flocks varies considerably among different reports, with Wedderkopp et al (2001) reporting a *Salmonella* prevalence of only 5.5%, while Arsenault et al (2007) reported a prevalence of 50%, Hayes et al (2000) reported a prevalence of 55.8%, and Santos et al (2005) reported a prevalence of 70-79%. The differences are likely due to the reports originating from different parts of the world and differing management practices. Although the studies utilized differing sampling techniques (drag swabs, cecal content samples, and litter samples), it has been shown that cecal content microbiota is related to litter microbiota (Cressman et al, 2010). The lowest, a 5.5% prevalence from Wedderkopp et al (2001) originated from Danish broiler flocks where they have implemented a pre-harvest *Salmonella* control program, while the other three reports

originated from North America where no such program exists (Hayes, et al, 2000;Santos et al, 2005;Arsenault et al, 2007;Wedderkopp et al, 2001) .

It has been found that the prevalence of *Salmonella* within poultry litter correlates well with *Salmonella* contamination within the cecum and in the finished poultry product (Campbell et al, 1982; Volkova et al, 2009; Cressman et al, 2010). Cressman et al (2010) conducted a study comparing the microbiota of commercial broiler litter and the of broiler intestinal contents. They found that poultry litter conditions significantly affect the microbial populations of the broiler intestines, and that intestinal flora of birds raised on clean litter as opposed to reused litter contained more bacteria of litter material origin. On the other hand birds raised on re-used litter contained bacterial populations of intestinal origin, thought to arise from previously present broiler flocks (Cressman et al, 2010). Campbell et al (1982) found that turkey carcasses from grower environments that had controls for *Salmonella* in place had significantly lower incidences of *Salmonella*, while turkey carcasses from grower environments without controls in place had significantly higher incidences of *Salmonella* positives (Campbell et al, 1982). In a comprehensive study of the farm to chiller continuum conducted by Volkova et al (2009), it was found that *Salmonella* positive carcasses post immersion chill tank were most associated with *Salmonella* positive litter samples from the grower houses taken on the day of harvest and prior to flock placement (Volkova et al, 2009). With these studies in mind, it is clear that preventative measures taken prior to the processing plant for control of foodborne bacterial contamination would be beneficial and that environmental and litter control could be effective critical control points within a poultry integrator's best management practices (BMPs).

There have been many studies performed to evaluate the sources of *Salmonella* contamination within poultry houses and on pre-harvest *Salmonella* control. These included competitive exclusion, litter acidification, vaccination, testing protocols, lactic acid administration, alternative litter materials, litter beetle control, and moisture and water activity control (Hoover, et al, 1997; Hayes et al, 2000; Mallinson et al, 2000; Pope and Cherry, 2000; Byrd et al, 2001; Eriksson de Rezende et al, 2001; Line, 2002; Skov et al, 2004; Line and Bailey, 2006; Payne et al, 2007; Al-Zenki, 2009; Torok et al, 2009). Hoover et al (1997) conducted a study to determine the source of *Salmonella* spp. colonization in two consecutive flocks of turkeys grown in newly constructed poultry houses. During this study litter, poult box liners, birds, drinkers, and air were all sampled from before the birds were placed at day 0 and at 2, 10, 14, and 18 weeks (2, 10, 14, 22 weeks for second flock); results of the study indicated that poults and feed were initial sources of *Salmonella* contamination within the house, indicating contamination of breeder flocks and/or the hatchery and of incoming feed shipments; results also indicated residual house contamination that further assisted in inoculation of incoming poults for the later flocks (Hoover et al, 1997). Although Hoover et al (1997) demonstrated potential sources of *Salmonella* contamination within newly constructed houses, their study concentrated on turkey flocks, did not quantify *Salmonella* populations throughout the flock cycles, while management practices and house design differed from that of commercial chicken broiler houses (Hoover et al, 1997).

Water activity and moisture content have proven to be useful parameters to adjust for decreasing *Salmonella* population in poultry litter (Hayes et al, 2000; Eriksson et al, 2001; Payne et al, 2007). Hayes et al (2000) concluded in their study that *Salmonella*

populations could be controlled by maintaining a litter A_w below 0.84 in conjunction with a moisture content between 20.0 and 25.0% through adequate ventilation (Hayes et al, 2000). Although their study did not quantify the *Salmonella* populations present within the litter, their findings are congruent with the results of Eriksson de Rezende et al (2001) and Payne et al (2007). Although the study by Eriksson de Rezende et al (2001) was an in vitro lab study their results were in agreement with the findings of Hayes et al (2000), indicating that maintaining a A_w below 0.85 and a relative humidity of less than 85% would decrease *Salmonella* populations within the surface litter (Hayes et al, 2000; Eriksson de Rezende et al, 2001). Payne et al (2007) modeled the rise and fall of *Salmonella* populations in respect to litter A_w and pH and concluded that ideal conditions possess a litter A_w below 0.84 and a pH $\leq$4 to effectively reduce *Salmonella* populations to below detectable limits. However, studies evaluating commercially available litter acidification products in a field setting did not prove to make a significant difference in *Salmonella* prevalence when compared to poultry houses that did not use the products, indicating that it may be relatively difficult to maintain a pH as low as 4.0 for any significant amount of time (Pope and Cherry, 2000; Line, 2002; Line and Bailey, 2006; Payne et al, 2007). The results of Pope and Cherry (2000) indicated that there was some on farm inhibitory effect on *Salmonella* and *E. coli* using Poultry Litter Treatment® (active ingredient: sodium bisulfate); however, there was no significant difference between treatment and control groups for presence of *Salmonella* in on farm bird rinses or drag swabs (Pope and Cherry, 2000). Similarly, two other studies evaluated the effects of two commercially available litter acidification products (aluminum sulfate and sodium bisulfate) and found that there was no significant difference between control and

treatment groups for *Salmonella* prevalence (Line, 2002; Line and Bailey, 2006). The litter acidification treatments in both studies though, did have an effect on *Campylobacter*. Line (2002) found that there was a significant difference in *Campylobacter* presence between treatment and control groups, while Line and Bailey (2006) found that litter acidification delayed the onset of *Campylobacter* colonization in broiler chicks (Line, 2002; Line and Bailey, 2006). The litter acidification products used in these studies were able to temporarily reduce litter pH soon after application; however, Line and Bailey (2006) reported that by week 2 the litter pH was approximately 6.0 and by week 4 the litter pH was not significantly different from the control litter (Line and Bailey, 2006). Line (2002) reported that the aluminum sulfate treatments maintained a lowered litter pH for a longer period of time than the sodium bisulfate; however, even the high dose treatments of aluminum sulfate only reduced the litter pH to 4.0 for a very short amount of time with litter pH reaching approximately 5.5 by week 1 (Line, 2002). Just as these studies concluded, litter acidification products may be beneficial to help lower the populations of some foodborne pathogens, but they should not be relied upon as the sole pre-harvest foodborne pathogen control measure.

Along the same lines as reducing litter pH, a study evaluating the effect of lactic acid administration in drinking water during pre-slaughter feed withdrawal on *Salmonella* and *Campylobacter* populations was performed (Byrd et al, 2001). This study revealed that the administration of lactic acid in the drinking water of broilers during pre-slaughter feed withdrawal significantly reduced the contamination of pre chill carcasses with *Salmonella* and *Campylobacter* species, making this yet another method of pre-slaughter control (Byrd et al, 2001).

Another avenue to consider in controlling *Salmonella* contamination in broiler chickens, that can be easily administered through the feed or sprayed on the chicks, is the use of probiotics for competitive exclusion purposes. In a study to evaluate the effectiveness of three commercial competitive exclusion and probiotic products (Aviguard, Levucell SC, and Bactocell) on *Salmonella* populations in broilers, it was found that all of these products were able to significantly reduce the *Salmonella* concentrations on the outside of the bird, in the ceca, and on the carcasses as compared to the control group without producing any adverse effects in regard to bird health or production standards (Al-Zenki et al, 2009). Further support of the use of competitive exclusion as a means to control the populations of pathogenic bacteria can be found in other dissimilar studies concentrating on the reuse of broiler litter for multiple flocks (Thaxton et al, 2003; Roll et al, 2011). These studies were aimed primarily at establishing the safety of litter used through multiple flock cycles due to concern over whether or not pathogenic bacterial species would accumulate within the litter over time to dangerous levels. However, Thaxton et al (2003) found that once the litter microflora was established the populations remained relatively stable regardless of how many birds were previously housed on it or by how many flock cycles had elapsed (Thaxton et al, 2003). Roll et al (2011) specifically evaluated the prevalence of *Salmonella* spp. in reused litter and found that reusing litter significantly reduces *Salmonella* contamination as a result of competitive exclusion and increased immunity due to exposure of low levels of microbial populations in reused litter at chick placement (Roll et al, 2011). Torok et al (2009) also found there to be a significant difference in cecal microbiota of chickens

raised on reused litter as opposed to fresh litter, taking into account different litter types (Torok et al, 2009).

Skov et al (2004) discovered that litter beetles may play a role in *Salmonella* transmission between consecutive flocks, although they did not find a link between litter beetles and *Campylobacter* transmission between flocks (Skov et al, 2004). Spraying for reduction of litter beetles between flocks may be an additional pre-harvest *Salmonella* control measure to consider along with other steps taken to implement a multimodal approach to *Salmonella* reduction.

A pre-harvest *Salmonella* control program comprised of multiple components has been implemented and used successfully in Denmark through incentives to farmers for growing "*Salmonella* free" birds, implementing rules that prohibit marketing of broiler chicken within the country that did not meet the target *Salmonella* goals, implementing a government compensated testing and depopulation program of infected breeder flocks, removal of organic material, thorough cleaning, and a rest period of 10-14 days between flocks (Wegener et al, 2003). Although this intensive pre-harvest *Salmonella* control program has been successful in Denmark, it would most likely not be feasible for a country that produced large quantities of broilers. The cost of the intensive testing programs coupled with compensation for depopulated flocks would be very costly; also, the lost earnings by producers who had to export their product to lower paying markets because of high *Salmonella* levels that did not meet target guidelines would be substantial. The program overview by Wegener et al (2003) did however, highlight the importance of a multi pronged approach to achieving lower populations of foodborne pathogens in the pre-harvest segment of the production chain to improve food safety.

One final concern associated with poultry production and food safety is the potential presence of pathogens that are antibiotic resistant. Kelley et al (1998) evaluated the presence of antibiotic resistance of bacterial isolates and found a high percentage of multiple antibiotic resistant litter isolates, although this was before the widespread usage of antibiotics in feed was reduced by the poultry industry (Kelley et al, 1998). Brooks et al (2010) also surveyed antibiotic resistance within a commercial poultry house and found that most of the predominant staphylococci were not antibiotic resistant or were at least susceptible to most antibiotics; however, most coliforms were resistant to 2 or more classes of antibiotics (Brooks et al, 2010). Further reduction or elimination of the usage of antibiotics for growth promotant purposes in the poultry industry in addition to practices already outlined that reduce pathogen populations within the poultry environment would serve to reduce contamination with antibiotic resistant strains of poultry associated pathogens.

Regulations/HACCP

Hazard Analysis Critical Control Point (HACCP) was implemented in most processing plants on Jan 26, 1998 and was implemented in all processing facilities by 2000. HACCP is a food safety program designed to identify, evaluate, and control potential hazards associated with the food supply; these hazards may be categorized as physical, chemical or biological in nature, and any process step where these hazards may be controlled is called a critical control point (FDA, 1997). HACCP is based on seven principles that are applied to the situation in order to design and implement a specific HACCP plan; those principles are as follows: conduct a hazard analysis, determine the

critical control points, establish critical limits, establish monitoring procedures, establish corrective actions, establish verification procedures, and establish record-keeping and documentation procedures (FDA, 1997). HACCP was designed for use from harvest to consumption; pre-harvest sectors are not required to implement HACCP protocols, but instead use best management practices to ensure food safety. Testing for *Salmonella* and *Escherichia coli* in processing facility environments and on bird carcasses is mandated by federal law. *Salmonella* prevalence in broiler carcasses before HAACP implementation (1994-1995) was reported by the USDA-Food Safety Inspection Service to be approximately 20%; similar data collected by the USDA-FSIS between July 1, 2011 and September 30, 2011 indicated *Salmonella* prevalence in broiler carcasses from all processing facilities to be 8.5% (USDA FSIS, 1995; USDA FSIS, 2011). This large reduction in *Salmonella* positive carcasses demonstrates the effectiveness of HACCP protocols.

Conclusion

In closing, *Salmonella* is a major foodborne pathogen of concern for the poultry industry with *Salmonella* causing approximately 23,128 hospitalizations with 452 annual deaths (Scallan et al, 2011). There are numerous pre-harvest methods of reducing final *Salmonella* contamination on broiler carcasses; however, it is most beneficial when a multi-modal approach targeting differing aspects of production is utilized. Presence of *Salmonella* within broiler litter has been established to be significantly associated with the presence of Salmonella on broiler carcasses, thus working to reduce litter

contamination and understanding dynamics of microbial population should be of great value (Volkova et al, 2010)

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CHAPTER II

SALMONELLA SOURCE AND RATE OF COLONIZATION IN TWO NEWLY CONSTRUCTED COMMERCIAL BROILER HOUSES AND THE EFFECT OF USED LITTER INOCULATION IN A NEW HOUSE

Introduction

The United States is the largest poultry producer in the world having a value of $34.7 billion in 2010; Mississippi is the fourth most productive state with an estimated value of $2.4 billion in 2011 (USDA-ERS, 2009; Collins-Smith, 2011; US Poultry and Egg Assoc, 2012). The annual cost of *Salmonella* infections in the United States is estimated to be 2.8 billion dollars with approximately 23,128 hospitalizations and 452 mortalities (Adhikari, 2004; Scallan et al, 2011). Poultry products are a known source of *Salmonella* infections in humans and as such, the poultry industry has been the focus of much food safety research. Many studies have documented the presence of *Salmonella* within poultry environments and described the pattern of colonization over time (Hoover et al, 1997; Mallinson et al, 2000; Terzich et al, 2000; Altekruse et al, 2006; Volkova et al, 2009; Cressman et al, 2010; Roll et al, 2011). However, there are no studies investigating the onset, source, and level of *Salmonella* contamination within newly constructed broiler grow-out houses. One study provided an ecological survey to

determine the source of *Salmonella* contamination in two consecutive turkey flocks grown in newly constructed houses; however, that particular study was focused on turkey flocks, had differing management practices and house design than those of broilers, and it did not provide quantitative data on *Salmonella* populations in the houses over time (Hoover et al, 1997).

Several studies have demonstrated the microbial effects associated with poultry litter reuse over multiple consecutive flocks to document the effect on *Salmonella* populations and to confirm the safety of this practice (Thaxton et al, 2003; Roll et al, 2011). These studies however, were conducted using older poultry houses with previously established microbial populations. To the best of our knowledge there has not been a study documenting the effect of utilizing used litter in newly constructed broiler houses on microbial population and *Salmonella* status. Anecdotally, this method of mixing used litter with the clean bedding material of newly constructed houses has been used to lower morbidity and mortality in the first few flocks of birds, presumably due to a competitive exclusion mechanism within the guts of the chickens.

The objective of the current study is to determine environmental sources and rate of *Salmonella* spp. colonization in newly constructed commercial broiler houses, and to evaluate the effect of mixing used broiler litter into the clean litter environment of a newly constructed broiler house. This study will determine: 1) the rate in which the poultry environment becomes contaminated with *Salmonella* following construction; 2) possible sources for contamination; and 3) the effect of adding used litter to the clean house environment.

Materials and Methods

Experimental Design

Two newly constructed commercial broiler houses, located on an approximately 3 year old farm, containing a total of eight houses (two new, six approximately three years old) in Mississippi were selected as the study site. The farm grows out approximately 5-6 flocks per year. Samples were collected from May 2011 until November 2011 encompassing 3 consecutive flocks. Background samples of soil and litter were taken before the first flocks were placed, twelve from each of the two new houses (six litter, six soil). One house had clean rice hulls placed for litter, while the other incorporated litter from the other six on site broiler houses in with the clean rice hulls. The two experimental houses were designated as clean and inoculated, respectively. Once flocks were placed in each of the two houses, litter and fecal samples were collected at two week intervals (week 0 – shortly after placing chicks, week 2, week 4, and week 6) for each flock. On each sampling date, twelve composite litter samples, comprised of litter taken from five separate locations for each sample, were taken from each of the two houses, and six fresh fecal samples were taken from each of the two houses. Samples taken on each sampling date totaled thirty six.

Sample Collection

New gloves and booties were donned before entering each house for sample collection. Background samples of soil and bedding (6 soil and 6 bedding samples from each house) were taken before flock 1 chicks were placed. Soil was hard packed clay and

had to be chiseled for collection. Five surface litter samples (0-3cm deep) were taken with gloved hands along the broiler house wall, between water and feeder lines, and along the center section of the house to form a composite for each sample. This was done on each side of the house and on both the fan and brood ends of the house to total (n=12) litter samples per house. Fresh fecal samples (n=6) were collected via convenience sampling from each house via sterile cotton tipped swabs and immediately transferred to 5ml of sterile tryptic soy broth (TSB) (Neogen-Accumedia; Lansing, MI) polypropylene tubes. A schematic of the inoculated house with sampling sites indicated is given in Figure 2.1. The clean house sampling sites mirror those of the inoculated house (Figure 2.1).

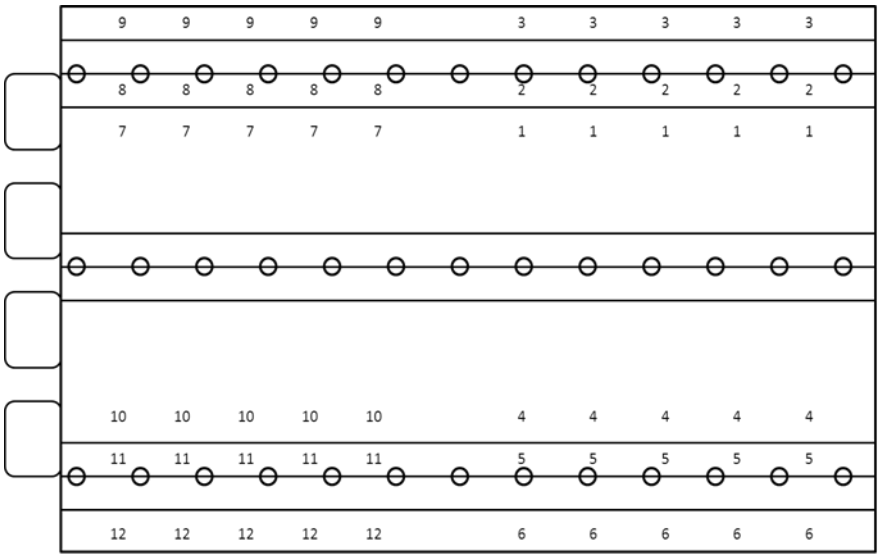

Figure 2.1
Schematic of broiler house depicting litter sampling points along the wall, between the feed and water lines, and along the center aisle. Sampling sites of second house mirror those of depicted house.

Microbial Assay

Microbial analysis for *Salmonella* spp. was performed as outlined by Brooks et al (2010). Litter samples were immediately transported to the lab and either prepared for a 3 dilution x 3 tube most probable number (MPN) analysis or for a presence absence analysis. Samples analyzed for presence/absence of *Salmonella* spp. were prepared by weighing 10 g of litter (wet weight) into a sterile bottle containing 95 ml of TSB (24h at 35° C) (Neogen), followed by transferring 0.5 ml of the vortexed sample to Rappaport Vasilidales R10 (RVR10) broth (Neogen) (24h at 42°C), and then transferring 0.1 ml x 3 of the vortexed sample to Rappaport-Vassiliadis Medium Semisolid Modified Agar (MSRV) (Neogen) plates for detection of motile *Salmonella* (24h at 42°C). MSRV plates that appeared positive as evidenced by a grey-white, hazy-turbid zone around the inoculated area were transferred to Hektoen Enteric Agar (HE) (Neogen) for confirmation (24h at 35°C). Isolates on HE that were pigmented blue-green with a black center were recorded as positive and an isolate was saved in 10% glycerol TSB at -20°C for later confirmation via polymerase chain reaction (PCR). Samples analyzed for MPN analysis were prepared by diluting 10 g (wet weight) litter into 95 ml sterile saline, stomached for 30 seconds, then aliquoted into the appropriate dilutions (0.1, 0.01, 0.001 g) or (0.01, 0.001, 0.0001 g) in 10 ml TSB (24h at 35°C). The MPN analysis then followed the approach of the presence/absence analysis. Fresh fecal samples were analyzed via the presence/absence method utilizing the 5 ml TSB tubes they were collected in. Moisture content was evaluated by weighing 10 g (wet weight) of the litter into pre-weighed aluminum tins and dried (104°C for 24-48h). The dried samples were reweighed and moisture content was calculated.

Colony polymerase chain reaction was performed on each of the *Salmonella* isolates to validate positive culture results. The saved isolates were streaked onto tryptic soy agar (TSA) (Neogen) and incubated at 35°C for 24 hours. Single colonies were lifted from the TSA and transferred to 1.0 ml of sterile PCR grade water and heated for 10 minutes at 99°C to lyse the cells before centrifuging at 5,000 rpm for 10 minutes. 0.1 ml of the supernatant was then transferred to 0.9 ml of sterile PCR water for dilution prior to PCR amplification. A master mix comprised of 1 X PCR Buffer II (Applied Biosystems; Foster City, CA), 2.5mM L^{-1} $MgCl_2$ (Applied Biosystems), 0.2mM L^{-1} dNTP mixture (Promega; Madison, WI), 1.5U AmpliTaq Gold (Applied Biosystems), bovine serum albumin (Fisher Scientific; Pittsburg, PA) sterile PCR water, and 200nM L^{-1} invA primers (Integrated DNA Technologies; Coralville, IA) was used with 10 µl of the diluted supernatant for the PCR assay using cycling conditions described by Liu et al, 2002. The finished product was assessed by electrophoresis on a 2% agarose gel, which was visualized by staining with ethidium bromide and photographing with an Alphatech gel imager (Alpha Innotech; San Leandro, CA).

Statistical Analysis

Prior to performing statistical analyses, all *Salmonella* spp. MPN values were $\log_{10}$ transformed to achieve normal distribution and to calculate geometric means. The effects of litter inoculation, bird age, flock number, sample location and their interactions on *Salmonella* spp. MPN values were investigated with one-way ANOVA and PROC MIXED analyses in SAS Enterprise Guide 4.2. Pairwise differences amongst means were tested with the Fisher's Least Significant Difference t-test. A Chi-Square table analysis

was used to determine the effect of inoculation and bird age on the rate of *Salmonella* presence/absence in litter and fecal matter. Unless otherwise stated an α value of 0.05 was used.

Results and Discussion

Salmonella Establishment and Environmental Sources

Presence/absence data from collected background samples of soil and rice hulls taken before chick placement revealed no *Salmonella* positive soil samples (data not shown). Only two *Salmonella* positive bedding samples out of six in the inoculated house and four *Salmonella* positive bedding samples out of six in the clean house were identified. The positive bedding samples were thought to have come from cross contamination from traffic through the houses, equipment, etc. Weather conditions during construction and preparation of the houses were very wet as well, which could exacerbate cross contamination. The lack of *Salmonella* within the soil samples indicates that the environment in which the houses were built did not likely contribute to *Salmonella* contamination within the houses. It is also of note that removing the top layer of soil during dirt work, pre-construction, may have assisted in removing any *Salmonella* from the soil environment.

Presence/absence data collected on litter and fresh fecal samples at two week intervals from three consecutive flocks revealed a sharp increase in *Salmonella* positive samples within the first two weeks of introducing birds into the new houses (Figure 2.2). Data from the second and third flocks indicate only slight population fluctuations

following establishment of *Salmonella* colonization (Figure 2.2), which is in agreement with Thaxton et al (2003) which reported that microbial populations tend to remain stable once established no matter the number of birds raised on the litter. The sudden increase in *Salmonella* positive samples following bird introduction suggests that contamination likely was introduced with the birds, although feed and water samples were not collected and remain a possibility for microbial contamination (Jones et al, 1991; Henken et al, 1992; Angen et al, 1996; Hoover et al, 1997; Rose et al, 1999; Chadfield et al, 2001; Corry et al, 2002).

The microbial flora of the gastrointestinal tract of chickens has been extensively studied by using both traditional culture methods and molecular methods, both finding that the gut microflora of newly hatched chicks is highly dependent on their surrounding environment and the feed and water they consume (Smith, 1965; Mead and Adams, 1975; Barnes et al, 1980; Coloe et al, 1984; Apajalahti et al, 2001; Lan et al, 2002; van der Wielen et al, 2002; Xiang et al, 2002; Hume et al, 2003; Jiangrang et al, 2003; Zhu and Joerger, 2003; Amit-Romach et al, 2004; Lan et al, 2005). Traditional cultural techniques have indicated that it takes approximately 2 weeks for microbial population establishment of the small intestine of broiler chicks, while it takes approximately 6-7 weeks to fully colonize the cecum (Smith, 1965; Coloe et al, 1984; Lan et al, 2005). Although cultural and molecular techniques differed on which organisms were predominant in the gastrointestinal (GI) tract of chickens, they agreed that microbial populations increase and become more variable with bird age (Smith, 1965; Coloe et al 1984; van der Wielen et al, 2002; Lan et al, 2005). *Salmonella* contamination of chicks in the present study most likely originated with the broiler breeder flocks or within the hatchery the supplied

the broiler grow out farm, both being previously reported as sources of contamination by other authors (McGarr et al, 1980; Keller et al, 1995; Angen et al, 1996; Christensen et al, 1997; Byrd et al, 1999; Skov et al, 1999; Dórea et al, 2010).

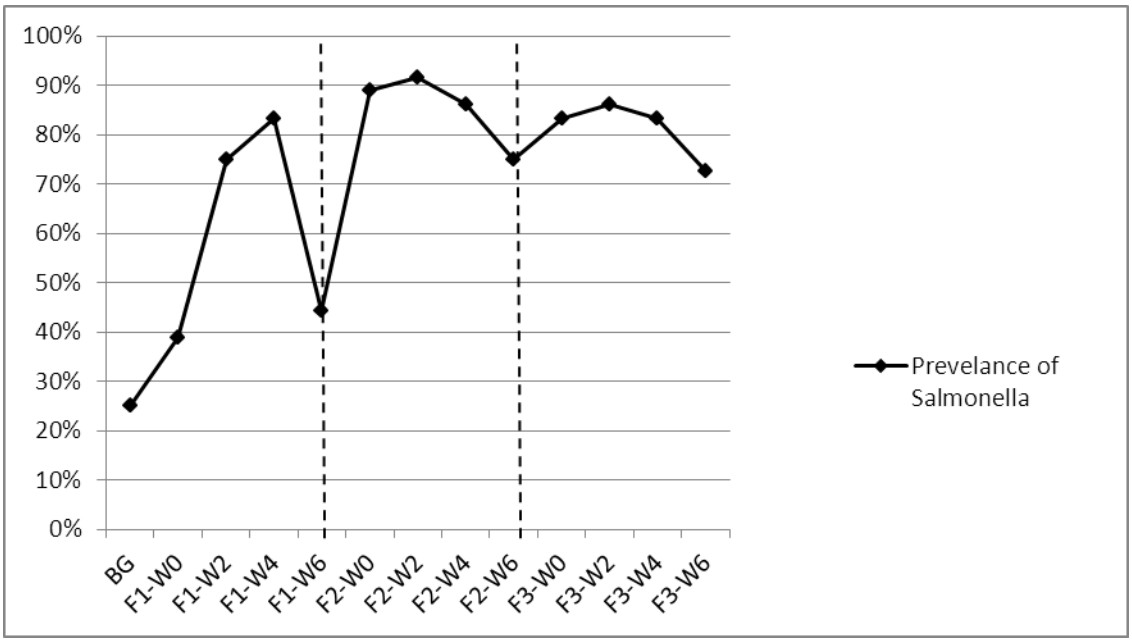

Figure 2.2

Prevalence of *Salmonella* positive litter and fecal samples throughout 3 consecutive flocks. BG=Background, F1=Flock 1, F2=Flock 2, F3=Flock 3, W0=Week 0, W2=Week 2, W4=Week 4, W6=Week 6. Dashed vertical lines represent breaks between flocks.

MPN data taken from all three flocks was compiled to form specific time point data; all three flocks had MPN data taken from the 4 and 6 week time points, flocks 2 and 3 had MPN data taken from the 2 week time point, and only flock 3 had MPN data taken from the 0 week time point. Sampling time points in which MPN analysis was not performed had presence/absence analysis performed instead due to expected low

population numbers. Statistical analysis using ANOVA revealed significant differences (α=.05) in *Salmonella* concentration per gram between Weeks 0 and 2, Weeks 0 and 4, Weeks 2 and 6, and Weeks 4 and 6; there was no significant difference in *Salmonella* concentration per gram between Weeks 0 and 6 or between Weeks 2 and 4 (Figure 2.3). These results indicate that there is a significant increase in litter *Salmonella* populations between placement of chicks at Week 0 and sampling at Week 2; *Salmonella* populations remain high between Weeks 2 and 4, which is why there was no statistical difference between these two time points. Populations at Week 6 drop back down to approximately what they were at Week 0 when chicks were placed, as indicated by a statistical difference between Week 4 and 6 and no significant difference between Weeks 0 and 6.

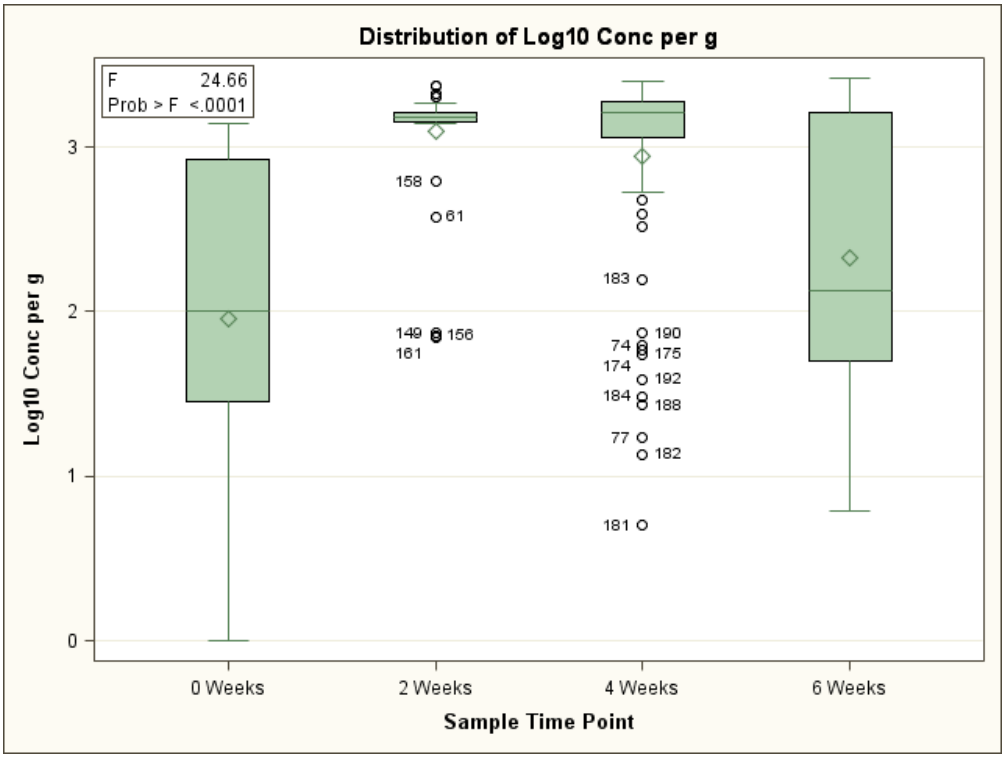

Figure 2.3
Box plot of MPN data from 3 consecutive flocks collected from both inoculated and clean houses.

Presence/absence data related to the litter from the same time points revealed an 80% *Salmonella* prevalence at Week 0, which increased to 97% and 100% in Weeks 4 and 6, respectively and dropped back down to an 85% *Salmonella* prevalence in Week 6 (Figure 2.4). Fecal samples taken during these same time points and analyzed for presence/absence of *Salmonella* also revealed the same trend of *Salmonella* presence as the litter samples, although fewer of the fecal samples were positive (Figure 2.4). These results may indicate that the litter samples were more sensitive for *Salmonella* detection than the fecal samples, perhaps due to the litter samples being composited. These results

could also indicate that the fecal samples represent more of real time prevalence, while the litter samples represent a delayed prevalence.

The fast rate of colonization between Week 0 and Week 2 may result from the immature gut microbiota of the new chicks (i.e. the normal gut microflora is not yet established), which leaves the chicks susceptible to *Salmonella* colonization and subsequent shedding (Barnes et al, 1972, 1980; Mead and Adams, 1975; Hoover et al, 1997; Bailey, 1998; Torok et al, 2009; Roll et al, 2011). Although it has been reported that it takes approximately 2 weeks for microbial establishment within the small intestine of the broiler chick, the most abundant microbe detected via molecular methods in chicks less than 14 days of age, has been reported to be *Lactobacillus* (Smith, 1965;Coloe et al, 1984; Amit-Romach et al, 2004). Low chick immunity to *Salmonella* may also plays a role in the quick establishment of *Salmonella* contamination in a poultry house; the *Salmonella* levels remained high from Week 2 through Week 4, but quickly declined to approximately the same level seen at Week 0, which may indicate that the bird's immune system and gut flora have matured enough to ward off and exclude *Salmonella* (Barnes et al, 1972, 1980; Mead and Adams, 1975; Corrier et al, 1992, 1993; Hoover et al, 1997; Bailey, 1998; Santos et al, 2005; Torok et al 2009; Roll et al, 2011).

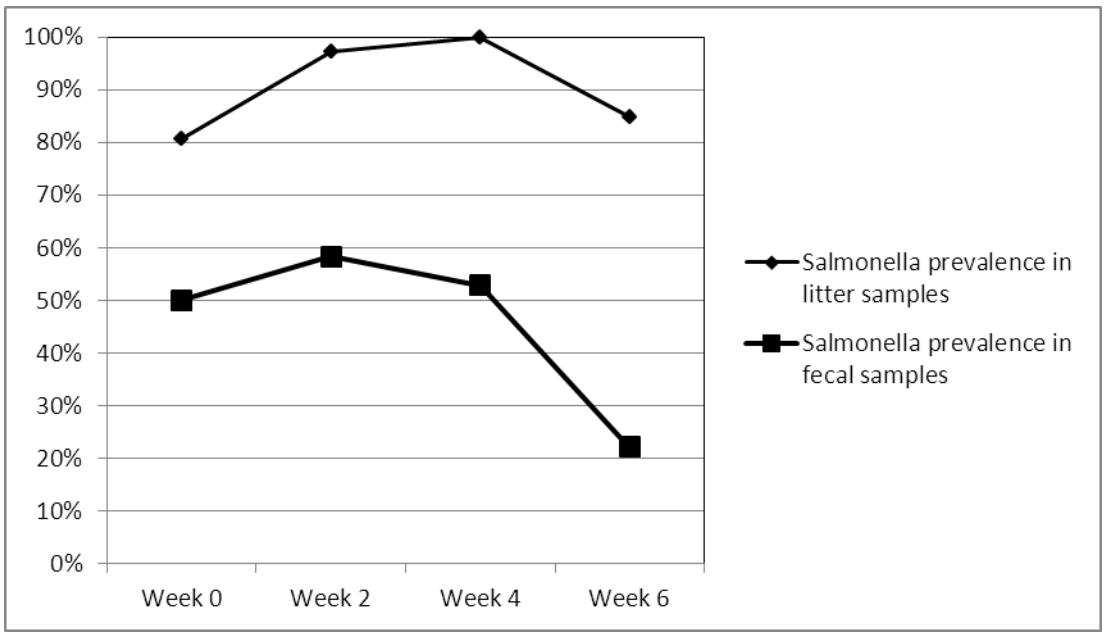

Figure 2.4
Salmonella prevalence in litter and fecal samples by time point
(data from 3 flocks compiled)

There were no significant findings associated with moisture content, sample site (next to wall, between feed and water lines, or center aisle), or house end (brood end vs fan end) as related to *Salmonella* prevalence, although moisture content was higher from samples taken from between the feed and water lines where birds tend to congregate. It had been previously reported that water activity (A_w) levels above 0.84 are favorable for *Salmonella* growth; however, *Salmonella* prevalence was high throughout the houses regardless of moisture content. Some authors have indicated the presence of *Salmonella* "hot spots" within poultry houses resulting from unevenly distributed environmental factors, such as A_w and pH, which could indicate drag swabs as being a more sensitive sampling approach (Hayes et al, 2000; Mallinson et al, 2000). However, the current

results did not follow this trend. *Salmonella* contamination in the current study site suggests dissemination throughout, showing no significant association with sample site.

Salmonella Prevalence in Inoculated vs Clean Houses

Presence/absence data collected throughout 3 consecutive flocks comparing the inoculated house to the clean house indicate a significant difference in *Salmonella* populations for the first flock, a slight non-significant difference for the second flock, and no significant difference for the third flock (Figure 2.5). This data suggests that mixing used litter in with the clean bedding in a newly constructed broiler house aids initially in reducing the *Salmonella* contamination within the house environment. However, this effect is short lived as the *Salmonella* population is established and remains stable in subsequent flocks. It should be noted that this part of the study only used two houses to compare the difference between inoculated and clean litter, due to their availability as the only newly constructed commercial houses in the vicinity for use in the study. A larger study using more houses would lend statistical support to this finding, although it does appear that *Salmonella* prevalence is initially reduced within the house as a result of this practice.

The lower *Salmonella* prevalence in the inoculated house, as opposed to the clean house, for the first flock data point is possibly explained as a consequence of competitive exclusion within the birds as well as within the litter, resulting in lower *Salmonella* prevalence in the house environment. The effect is quickly diminished, however, when populations level out with subsequent flocks. This diminishing effect is most likely seen because chicks from subsequent flocks are being immediately exposed to large

populations of *Salmonella* in the litter from the moment of placement within the houses when they are most susceptible to the pathogen due to natural low immunity (Milner and Shaffer, 1952). In order to prevent *Salmonella* colonization within newly constructed broiler houses it is imperative to ensure that the environment, feed and water supply, and broiler breeder flocks and hatcheries that supply the new houses are free of *Salmonella* contamination. If it is known that one of these sources is already contaminated with *Salmonella*, then it may be initially beneficial to inoculate the new houses with used litter to provide a source of competing microbes.

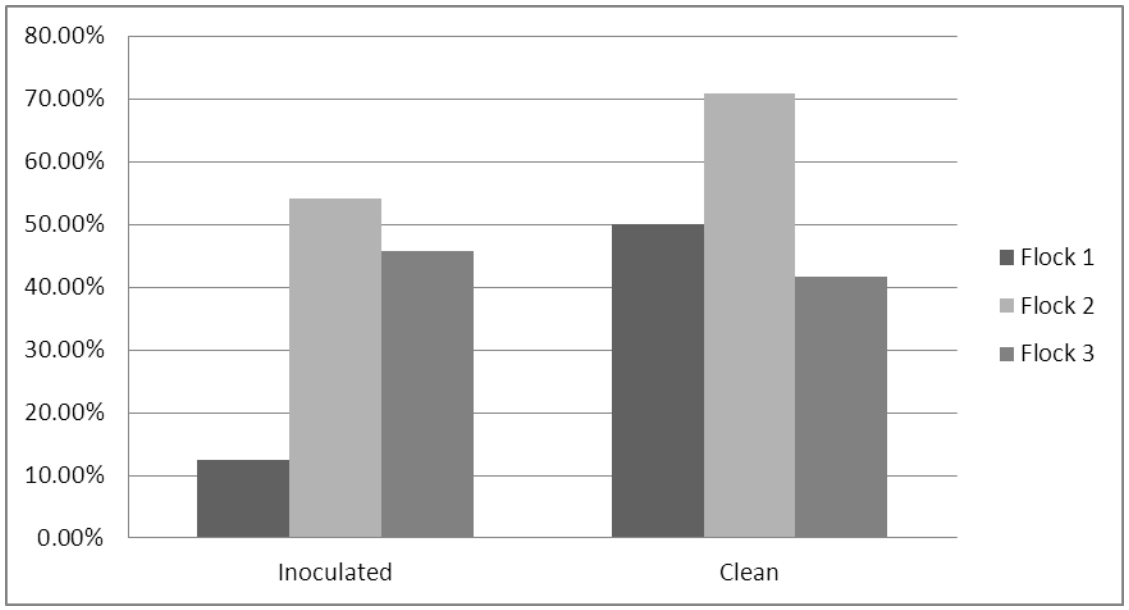

Figure 2.5

Salmonella prevalence throughout three consecutive flocks: clean house and inoculated house. Inoculated house (House 7) had used litter mixed with clean rice hulls. Clean house (House 8) had clean rice hulls.

Conclusion

In conclusion, the results of this study illustrate the need for a multi faceted approach to pre-harvest best management practices to improve food safety by decreasing the prevalence of *Salmonella* within poultry flock environments and thus decreasing *Salmonella* contamination in processing facilities. Although anthropogenic contamination cannot be ruled out completely because it is known that some contamination likely occurred from movement of people between houses, contamination in these newly constructed houses likely arose from the newly placed chicks via contaminated broiler breeder flocks and hatcheries, from which the chicks originated. Reducing *Salmonella* populations within these segments of the production chain have been shown to reduce *Salmonella* prevalence within processing facilities (Vokova et al, 2009; Dórea et al, 2010). This work showed *Salmonella* populations are at their highest level during weeks 2-4, but decline to pre chick levels by week 6. This *Salmonella* prevalence fluctuation cycle lends support to the safety of reusing litter for multiple flocks instead of completely cleaning the houses out after each flock. Finally, the use of inoculated litter in newly constructed houses may help reduce *Salmonella* contamination in the first few flocks raised in the houses if it is known that the chicks being placed are *Salmonella* positive. Studies evaluating additional new houses are needed to lend statistical impact to these findings.

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