8-1-2010

Prevalence of Campylobacter jejuni in newly constructed broiler houses

Krista Nicole Eberle

PREVALENCE OF *CAMPYLOBACTER JEJUNI* IN NEWLY CONSTRUCTED BROILER HOUSES: A ONE YEAR SURVEY

By

Krista Nicole Eberle

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agricultural Science
in the Department of Poultry Science

Mississippi State, Mississippi

August 2010

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Krista Nicole Eberle

Approved:

Aaron S. Kiess Assistant Professor of Poultry Science (Director of Thesis)	Christopher McDaniel Professor of Poultry Science (Committee Member)
Jeremiah Davis Assistant Professor of Ag & Bio Engineering (Committee Member)	Jody Purswell United States Department of Agriculture Agricultural Research Service Agricultural Engineer (Committee Member)
Christopher McDaniel Professor of Poultry Science Director of Graduate Studies in the Department of Poultry Science	Melissa Mixon Interim Dean of the College of Agriculture and Life Sciences

Name: Krista Nicole Eberle

Date of Degree: 37BAugust 2010

Institution: Mississippi State University

Major Field: Agricultural Science

Major Professor: Dr. Aaron S. Kiess

Title of Study: PREVALENCE OF *CAMPYLOBACTER JEJUNI* IN NEWLY CONSTRUCTED BROILER HOUSES: A ONE YEAR SURVEY

Pages in Study: 89

Candidate for Degree of Master of Science

Campylobacter is the leading cause of bacterial gastroenteritis in the United States. The objective of this study was to investigate the prevalence of *Campylobacter* in newly constructed broiler houses and compare three microaerophilic gas delivery methods used to culture *Campylobacter* in the laboratory. Of 2,300 litter, 900 fecal, and 45 water samples, only 5, 6 and 1 of the samples, respectively, were confirmed positive. Results indicated litter moisture content was different across day, location and house. An interaction was detected for litter pH between day, location and flock. Temperatures averaged 26.8°C inside and 27.6°C outside. No difference in colony counts were detected among the gas delivery methods. In conclusion, the newly constructed houses showed no significant prevalence of *Campylobacter*. High litter pH, low temperatures, and other on-farm management strategies may have suppressed *Campylobacter's* ability to colonize the litter. When selecting a gas delivery method price and space should be considered

DEDICATION

I would like to dedicate this work to my loving and supportive parents, Rick and Mary Jo Eberle.

ACKNOWLEDGEMENTS

The author expresses her heartfelt gratitude to everyone who made this research possible. First and foremost, a special thank you is due to my major professor Dr. Aaron Kiess for his patience and guidance throughout my masters study as well as his encouragement and support as I start down a promising career path. Thank you to the committee members Dr. Chris McDaniel, Dr. Jeremiah Davis, and Dr. Jody Purswell. Thank you to my "lab monkies" Walt Stephens and Melissa Haines, whose generous help, hard work, unwavering friendship and laughter made early mornings and late nights seem like a day in the park. Thank you to Stacy Maher for always being such a giving friend. I could not have made it through some of the hard days without you. Thank you to my uncle and aunt, Carl and Deb Koebbe, for your generosity and for allowing me to make a home with you in Georgia. Lastly, to my friends and family back in Indiana, I could not have made it through the past two years without your reassurance and love, and for that I am eternally grateful.

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

As the incidence of foodborne illness have increased, so has consumer awareness and concern of food safety. An estimated 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths occur from foodborne diseases in the United States every year (CDC, 2008). Of the four types of disease-causing pathogens—viruses, bacteria, fungi and parasites—bacteria account for over 90% of confirmed foodborne infections and deaths reported to the Center for Disease Control (Buzby et. al., 1996). Of the five most prevalent foodborne bacteria (*Campylobacter*, nontyphoidal *Salmonella*, *E. coli* O157:H7, *E. coli* non-O157 STEC and *Listeria monocytogenes*), *Campylobacter* spp. are the most frequent cause of bacterial gastroenteritis in the United States (Newell and Fearnley, 2003). While humans have been reported to acquire *Campylobacter* from exposure to unpasteurized milk (Yang et. al., 2003; Schildt et. al., 2006; Heuvelink et. al., 2009), contaminated water, meat, or vegetables (Knill et. al., 1981; Kumar et. al., 2001; Evans et. al., 2003), the main route of procuring this bacterium is through the ingestion of raw or undercooked poultry (Mehle et. al., 1981; Stanely et. al., 1995; Little et. al., 2008). As the number of farmers in the United States decreases with modernization of equipment, genetics, and management techniques as well as the decrease in land availability, consumer reliance on food manufacturers to provide safe products has increased. The yearly consumption of poultry by the average American in 1980 was 48.9 pounds. Poultry consumption increased 35% from 1982 to 1992 (Lin et. al., 1993).

By 2007, the consumption of poultry and poultry products was 86.3 pounds per person per year (American Meat Institute, 2009). This increase in poultry consumption may have been due to the following factors: the substitution of chicken for other meats as chicken became more affordable, an increase in chicken availability in the supermarket, and an increase of low fat and high protein diets (Buzby et. al., 1996).

The increase of consumer dependence on the development of safe products by food manufacturers and the increase of poultry consumption has resulted in the development of food safety regulations, particularly in the meat and poultry processing plants. In July of 1996, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) implemented the Pathogen Reduction and Hazard Analysis and Critical Control Point (HACCP) Systems final rule with the goal of improving food safety. In this rule, USDA-FSIS established standards designed to reduce the occurrence and levels of pathogenic organisms on meat and poultry products in addition to reducing the incidences of food-borne illness associated with the consumption of those products (USDA-FSIS, 1996). This rule also discussed a farm-to-table strategy for the control of food safety hazards throughout the entire process of animal production. While *Salmonella* standards have been developed and enforced, *Campylobacter* standards have yet to be refined due to the lack of data for *Campylobacter* levels on raw poultry carcasses. In 2008, FSIS stated that they are currently developing a baseline for *Campylobacter* levels within the processing plant (USDA-FSIS, 2009). Consequently, in 2009 FSIS announced the development of new pathogen reduction performance standards for *Salmonella* and *Campylobacter* both on-farm as well as in the processing plant (USDA-FSIS, 2009). The agency expects that by reducing the number of bacteria on the farm it can significantly lower the possibility for contamination in the plant. Also,

evidence of bacterial levels on-farm will help indicate which farms would be better to obtain birds from and even provide information for the development of a priority slaughter schedule for producers harboring low *Salmonella* and *Campylobacter* levels (USDA-FSIS, 2009).

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CHAPTER II

LITERATURE REVIEW

History of Broiler Production

In the early part of the 20th century, most chickens were raised in small backyard flocks where meat was considered to be a derivative of egg production. Farmers would "set" eggs or buy chicks locally to grow-out, selling the cockerels for meat and keeping the hens to lay eggs (Sawyer, 1971). It wasn't until the late 1920's that the concept of raising poultry year round in enclosed, controlled environments strictly for meat consumption became a reality (Connor, 2008). The term "broiler" defines chickens of meat-type genetic strains that are raised specifically for the meat market (Agricultural Statistics Board, 2002). These meat-type genetic strains were developed in the late 1940's and are still in use today (Sawyer, 1971). As the availability and demand for chicken meat began to grow in the 1950's, production was shifted from home-based production to larger commercial operations. The southern states, because of their warm climates, poor soil and uneven landscape became the optimal region for these large establishments. Before World War II, farmers would purchase baby chicks from local hatcheries typically supplied by their own breeding farms. Feed was bought at local feed mills or at feed stores where grain was shipped from the Midwest. After rearing, chickens would then be sold at market size (around 2.5 pounds and 12-16 weeks of age) to local buyers, brokers, or through auctions. The stability of the market was imbalanced due to fluctuation of the supply of baby chicks vs. the demand of feed (Martinez, 1999). This

economic instability lead to the concept of "vertical integration", in which all the separate components of the production process—breeder farm, hatchery, feed mills, and eventually grow-out operations and processing plants—were brought together under one single company (Connor, 2008). By 1955, most growers had established contracts with integrators, both sharing responsibilities of risk and profit involved with poultry production. Growers would provide housing, equipment, labor, and fuel as well as other inputs while the company would provide the baby chicks and feed along with veterinary services and any needed supplemental information (Martinez, 1999). The development of vertical integration and contracting growers improved coordination throughout the stages of production as well as increased the efficiency and profitability of the company.

Since the establishment of grower contracts in the mid-1900s, the poultry housing environment in which broilers are raised has made drastic improvements. House designs have moved from conventional curtain-sided housing with natural ventilation to solid-sided housing with tunnel ventilation, increasing the uniformity and movement of air through the house (Czarick and Tyson, 1990). Foggers and evaporative cooling pads have also been added to help reduce air temperature during the hot summer months (Czarick and Lacy, 1991b). Changes inside and ceiling insulation materials and square footage have helped minimize heat gain in the summer and heat loss in the winter (Czarick and Lacy, 1991a). The use of radiant brooders and dimmed incandescent light bulbs has replaced air furnaces and 100 watt light bulbs to save energy (Czarick and Lacy, 1991a; Czarick and Lacy, 1994).

The broiler industry's transition into vertical integration has allowed for the incorporation and utilization of new technologies that have enhanced rearing and processing procedures, reduced costs and formed a single profit point which has allowed

the poultry industry to respond quicker to consumer demands (Mississippi State University Extension Service, 2009). By becoming vertically integrated, the United States is now recognized as the world's leading producer of broiler meat (Jordan, 2002).

Litter and Environmental Influences on Microbial Proliferation

Litter is defined as the combination of water, bedding material, excreta, feathers, and wasted feed. The ideal litter should be durable, contain little moisture and not readily clump, or cake (Swain and Sundaram, 2000). The bedding material serves several functions in the house: providing a protective cushion between the birds and the floor, insulating the chicks from cold weather, absorbing excess moisture from fecal matter and drinkers as well as diluting fecal material to reduce exposure to birds.

The type and quality of bedding is dependent on regional availability and costs. It is also important that the material selected is nontoxic to the birds (Butcher and Miles, 1995). Pine shavings and sawdust have been and continue to be the preferred source of bedding material, but as availability decreases and cost increases, producers are forced to use alternative forms of litter. Previous research has shown hardwood shavings and sawdust, pine or hardwood chips and bark (Carter et. al., 1978; Brake et. al., 1992), rice and peanut hulls (Anisuzzaman and Chowdhury, 1996; Lien et. al., 1998), chopped straw and hay (Benabdeljelil and Ayachi, 1996), crushed corn cobs, leaves (Willis et. al., 1997) and sand (Bilgili et. al., 1999) to be efficient alternatives (Ritz et. al., 2005). Each of these materials has strengths and weaknesses in utilization.

The reuse of litter is a common practice in most poultry companies due to the high cost and availability of bedding material as well as the difficulty of disposal of the material (Ritz et. al., 2005). Management of reused litter is crucial to help reduce not

only cost but to control pathogens that live in the litter and may cause disease to the flock. Several methods, including decaking, top dressing, and windrowing, have been shown to extend the life of the litter (Coufal et. al., 2006; Malone, 2008). When moisture in present on the litter and in the air in a house, litter can clump up, or cake. Cake can be removed by passing the litter over a grate, separating it from the dry litter (Coufal et. al., 2006). This management practice removes moisture and allows the litter to dry out further, creating an unfavorable environment for bacterial growth. Top dressing is a litter management method in which a thin layer of fresh litter is spread over the old litter prior to the addition of a new flock. This spreading of fresh litter helps increase the absorptivity of the old litter and discourages caking.

A recent trend, windrow composting or windrowing, uses heat and moisture to transform biodegradable waste in to a stable, odorless compost product with reduced levels of most pathogens (Macklin et. al., 2006). Typically, these windrowed piles can range in temperature from 57 ºC to 76 ºC, creating high enough temperatures to greatly reduce bacterial loads in the litter. Windrowed piles also reduce microbial counts using high ammonia levels as a result of the increase in temperature as well as the competitive exclusion by the growth of other microorganisms (Macklin et. al., 2008). Macklin and colleagues (2008) showed that in-house composting of litter is an effective way of reducing, and in some cases, eliminating, foodborne pathogens in a broiler house.

With Campylobacter's atmosphere and temperature requirements, recovery of this bacterium from litter has been difficult and because of that difficulty, not thoroughly explored. Montrose and colleagues (1985) were among the first to investigate litter as a potential source of Campylobacter transmission. They demonstrated that litter was not only a source of Campylobacter transmission to chicks, but that it could also persist in the

litter for more than 46 days (Montrose et. al., 1985). Kazwala and others (1990) also isolated *Campylobacter* from litter, finding it to be not the initial source of transmission, but a vector after chicks became infected. In 1992, van de Giessen et. al. found that *Campylobacter* may have the ability to spread through subsequent flocks (van de Giessen et. al., 1992). Cox and others (2001) have demonstrated *Campylobacter* recovery from dry poultry samples, including pine shavings, diminishes quickly over a short period of time. They also suggested that organic material, such as excreta, may provide protection for *Campylobacter* in dry conditions (Cox et.al., 2001).

Campylobacter growth on both new and reused litter and within a poultry flock can be influenced by several environmental factors, including temperature, pH, humidity and moisture. These factors are intertwined, frequently affecting the occurrence and intensity of one another. Environmental factors also vary at each location in a house (evaporative cooling pads and tunnel ventilation fans, water/feeder lines) which can have a major effect on the microbial dynamic within the litter (Lovanh et. al., 2007).

Research has shown that human campylobacterosis infections follow seasonal patterns that coincide with the prevalence of *Campylobacter* in broiler flocks (Wallace et. al., 1997; Jones, 2001; Patrick et. al., 2004). These seasonal trends suggest that temperature, sunlight hours, and relative humidity plays a major role in *Campylobacter* transmission. Patrick and others (2004) determined that maximum temperatures between 13 ˚C and 20 ˚C corresponded with an increase in human campylobacterosis infections as well as an increase in the percentage of infected broiler flocks at slaughter. Refregier-Petton et al. (2001), Jones (2001), Patrick et. al. (2004) and Kovats et. al. (2005) all showed peaks of human campylobacter infections in the spring, summer and early autumn months, where the average temperature and maximum temperature had little

variation. Wallace et. al. demonstrated higher levels of campylobacter in the small intestines and ceca of birds in February as well as the June, July, and August (Wallace et. al., 1997) Although the ideal growth temperature for *C. jejuni* is 42˚C, it has been found to grow in temperatures varying from 30˚C to 47˚C (Chan et. al., 2001; Stintnzi, 2002). Chan et. al. (2001) demonstrated that some poultry campylobacter isolates can even tolerate temperatures as low as 4˚C.

Moisture content in litter is essential for pathogen growth in a poultry house; the availability of water is one of the components that determine the survival of bacteria in poultry litter (Line, 2006). Wet litter can be brought about by several means. If litter is stored improperly in a damp environment before placement in a house, it is more likely that the litter will stay damp after being spread (Ritz et. al., 2005). While it is not possible to have control of the ambient temperatures and humidity, keeping proper temperature and humidity levels in the house during production is key to keeping litter moisture under control. Wet, cold or humid weather as well as condensation can lead to damp litter. Line (2006) found that a reduction of relative humidity led to a delay in *Campylobacter* colonization in birds raised on contaminated or used litter. Heating and ventilation also has an effect on litter moisture content. Monitoring of these systems are vital to control moisture content in the air and on the litter as well as ammonia levels in a broiler house. Leaky drinker systems and evaporative cooling pads can increase the litter moisture resulting in an increase of caked litter (Ritz et. al., 2005). By keeping all of these systems in good condition it is possible to reduce moisture content of the litter thereby lowering microbial growth.

Like temperature and moisture content, pH plays a vital role in determining the ability of bacteria to grow in a certain environment. Most bacteria, including

Campylobacter, prefer an environment with a pH range of 6.5 to 7.5; they can survive at a pH as low as 4.9 (Doyle, 1989). Acidifying litter treatments, commonly used to reduce ammonia levels in a house, have been found to also lower pathogen populations on the litter and in the intestines and ceca of broilers (Line, 2002; Garrido et. al., 2004). The most popular litter treatments are aluminum sulfate, or alum, and sodium bisulfate. These acidic litter treatments affect ammonia levels by shifting the NH_3/NH_4 equilibrium towards NH_4, effectively decreasing the pH and the water activity and creating a bacteriostatic or bacteriocidal environment for pathogens like *C. jejuni* (Line et. al., 2002; Choi et. al., 2008).

Reuse of litter in broiler houses is due to high costs and limited availability of materials. Durable litter and the utilization of good management techniques before, during, and after a flock can greatly reduce the pathogen load found in the housing environment and in the birds in addition to extending the life of litter. By keeping moisture content, ammonia levels and pH low through the proper maintenance of heating and ventilation systems and the use of litter treatments, an antagonistic environment for *Campylobacter* growth can be produced reducing the risk of litter colonization by *Campylobacter*.

Characteristics of *Campylobacter*

Campylobacter, a member of the family *Campylobacteriaceae,* is a pathogenic, vibrioid gram-negative bacterium generally found in the reproductive organs (Cox et. al. 2006), intestinal tract (Wallace, et. at. 1997), and oral cavity of humans and animals (Macuch and Tanner, 2000). In the genus *Campylobacter*, there are currently 17 species and 9 subspecies (Engberg, 2006). *C. jejuni* is the most common *Campylobacter* species

found in poultry. *Campylobacter* cells are slender, spiral-curved rods, ranging from 0.2-0.5 μm wide to 0.5-5 μm long. They are non-sporeforming, and when two cells form short chains, can appear S-shaped or gull-wing-shaped. When exposed to adverse conditions, these cells form a coccal shape, becoming viable but nonculturable (VBNC); this can decrease but not eliminate their pathogenicity (Fearnley et. al., 1995; Holt et. al. 2000). *Campylobacter* spp. move in straight lines with a corkscrew-like motion due to a single, unsheathed polar flagellum at one or both ends of the cell. This flagellum is long and can be several times the length of the cell.

Campylobacter spp. are typically microaerophilic, requiring an environment containing 3-15% O_2 and 3-5% CO_2 and a temperature of 37 °C to grow. Four of the 17 species—*C. jejuni*, *C. lari*, *C. coli* and *C. upsaliensis*—are thermophilic, requiring a slightly higher temperature of 42 °C. Some strains require H_2 or formate to grow microaerophilically, while others can grow in anaerobic conditions that contain fumerate, formate plus fumerate, or H_2 plus fumerate. A few strains are able to grow under aerobic conditions (21% O_2). *Campylobacter* is chemoorganotrophic with a respiratory type of metabolism, obtaining energy from amino acids or tricarboxylic acid cycle intermediates (Holt et al., 2000). *Campylobacter jejuni* hydrolyzes hippurate, indoxyl, and acetate and reduces nitrate, but is unable to oxidize or ferment carbohydrates (Keener et. al., 2004). They are urease negative and oxidase positive (Holt et. al., 2000).

Until 1963, *Campylobacter* spp. were originally part of the genus *Vibrio*; it was Sebald and Veron who differentiated campylobacters from the members of the genus *Vibrio* by their low G+C DNA content, microaerophilic requirements, and nonsaccharolytic, or "nonfermenting" metabolism (Dworkin, 2006). *Campylobacter* was not isolated from human feces, however, until ten years after its discovery by Dekeyser

(1972) and Butzler (1973). Due to the fragile nature and novelty of this microorganism, little interest was shown in Dekeyser and Butzler's discovery until 1977 when Skirrow (1977) developed an antibiotic supplemented blood-agar. This agar provided a way to successfully isolate campylobacters on a large scale, eventually denoting *Campylobacter's* capacity to cause human gastroenteritis (Skirrow, 1977).

Campylobacter spp. cause the infectious disease campylobacterosis. Symptoms of campylobacteriosis typically include headache, muscle pain, and fever that is followed by watery or bloody diarrhea, nausea and abdominal pain. These symptoms occur 2-5 days after ingestion and can last from 3-10 days (Keener, 2004). In one of 1000 cases, it also leads to a rare autoimmune disorder of the peripheral nervous system, known as Guillain-Barre syndrome (GBS), in which the affected person experiences a rapid decline in muscle strength in the limbs and respiratory system (Center for Disease Control, 2008, Nachamkin et. al., 1998, Keener, 2004). Other possible autoimmune disorders than can occur from *Campylobacter* infections include Miller Fisher syndrome and Reiter's syndrome, also known as reactive arthritis (Nachamkin et. al., 1998, Keener 2004).

Modes of *Campylobacter* Transmission

The main source for *Campylobacter* transmission to poultry is still unclear. Introduction of *Campylobacter* has been found to occur at all levels of poultry production, from the hatchery (Byrd et. al., 2007) to the farm (Shanker et. al., 1990; Jacob-Reitsma et. al., 1995) to the processing plant (Oosterom et. al., 1983; Wempe et. al., 1983; Corry and Atabay, 2001). Sources and routes of transmission of *C. jejuni* to broilers on-farm vary with location and management practices. Colonization first occurs in broilers between weeks 3-4 of age (Stern et. al., 2001). This initial colonization period

is important to consider because several studies have shown that with vigorous cleaning and disinfecting of poultry houses along with strict biosecurity measures, infection can be postponed (Humphrey et. al., 1993; van de Giessen et. al., 1998). Prevalence of *Campylobacter* in the processing plant is attributed to *Campylobacter*-positive birds coming from the farm (Oosterom et. al., 1983). *Campylobacter jejuni* has been found to colonize the lower part of the birds intestinal tract, principally the large intestine, ceca, and cloaca and can be found in concentrations as high as 10^7 per gram (Bryan and Doyle, 1994).

On-farm

To date, there is still some uncertainty surrounding vertical transmission of *Campylobacter* to broiler flocks. Smith et. al. (2004) conducted a study concerning *Campylobacter* colonization in sibling pairs of turkey flocks. Results demonstrated that vertical transmission did not occur or was not sufficient enough to show transfer of *Campylobacter*. Callicott et. al. (2006) sampled from parent flocks representing over 60,000 offspring; their results demonstrated that there was no evidence of *Campylobacter* colonization in offspring that were quarantined. It was not until the birds were moved into rearing facilities that any flocks were found positive for *Campylobacter* (Callicott, 2006). In contrast, Pearson et. al. (1996) proposed that *Campylobacter* transmission could be traced back to the hatchery. The study showed evidence of a common source originating from the parent breeder flocks through the vertical transmission pathway (Pearson et. al., 1996). These studies, while contradictory, showed no indication that vertical transmission was a significant mode of *Campylobacter* transmission, suggesting that an environment vector was responsible for the introduction of this organism.

Horizontal transmission is thought to be the more likely mode of transfer of *Campylobacter* among broiler houses. Contaminated feed, litter, water, routine and maintenance visits, veterinarian calls, and load out crews, cross-contamination from nearby houses, or unauthorized entry of wild animals, birds, and insects have all been implicated as possible sources of campylobacter infections on the farm (Newell and Fearnely, 2003).

The dry conditions of fresh, or unexposed, litter and feed are considered an unfavorable environment for *Campylobacter* growth (Pearson et. al., 1993; Newell and Fearnley, 2003). Jacob-Reitsma and colleagues (1995) were unable to detect *Campylobacter* in feed and litter samples taken from bins not yet in contact with birds. While *Campylobacter* may not be found in detectable levels in fresh feed or litter that does not mean that this bacterium is absent. The dry, stressful environment that feed and litter maintain may cause cells to enter a VBNC state; in this state, *Campylobacter* can remain potentially pathogenic (Tholozan et. al., 1999). Water sources, drinker lines, and drinkers have been suggested as locations for possible *Campylobacter* colonization in the house (Pearson et. al., 1993; Ogden et. al., 2007). Pearson and colleagues (1993) were not able to culture *Campylobacter* from any of the previously listed sources; they were, however, able to provide evidence that water is a potential source of transmission. By feeding separately raised *Campylobacter*-free flocks suspect farm water, they were able to observe shedding of the same *C. jejuni* serotype. Zimmer and others (2003) were able to visualize *Campylobacter* cells in biofilm found on drinker nipples but were not able to culture the cells, suggesting that the biofilm may present an environment for Campylobacter to persist, even in a VBNC state. Unless contaminated, it is unlikely

that unexposed feed, litter, and water are a prominent source of *Campylobacter* infection in broilers.

Wild rodents, birds, and insects along with domestic pets and livestock are often vectors for *Campylobacter*. These animals can carry the bacteria in their mouth and intestinal tracts, very rarely showing signs of infection. The spread of *Campylobacter* by the above vectors is usually through contact with infected fecal matter which is then transported into the housing environment. The degree of contamination and transmission of *Campylobacter* is dependent on the habitat, whether the broiler houses are in an urban environment, near woods, on a farm, etc. The type of production also makes a difference, as organic and free-range farms may have a higher risk of attracting these animals than a commercial farm would (Meerburg and Kijlstra, 2007). Mammals are generally regarded as the main environmental reservoir for pathogens like *Campylobacter*. Wild rodents are attracted to the availability of feed, water, and shelter that a broiler house provides. They acquire these diseases from the feces of other wild animals, livestock, or from each other since rodents tend to live in close proximity to one another. Livestock, including cattle, sheep, and hogs, and domestic pets have also been observed shedding *Campylobacter jejuni* within or in close proximity of the broiler house environment (Luechtefeld and Wang, 1981; Bruce, 1981; Stanely and Jones, 2003). *Campylobacter* spp. have been isolated from other avian species, especially migratory fowl like ducks and geese and scavengers like crows and pigeons (Fenlon et. al., 1981; Waldenstrom et. al., 2002). Wire mesh and other barriers are used to keep wild birds out of the houses. They can however, peck through the barriers and are able to enter the house (Craven et. al., 2000). Insects—like flies, beetles, and cockroaches—live in and around the houses and have been found to carry *Campylobacter*. Skov et. al. (2004) found that campylobacter

positive litter beetles were detected in connection with positive flocks. It was suggested by Templeton and colleagues (2006) that while darkling beetles are not long term carries of *Campylobacter*, after constant ingestion of infected litter, can be short term carriers of the bacteria. Flies, which enter houses through the ventilation systems, have been found to carry and shed campylobacter spp., especially during the summer months (Hald et. al., 2004). Flies forage on fresh feces from the surrounding environment and have demonstrated infection within two hours of *C. jejuni* ingestion (Shane et. al., 1984).

Campylobacter can also be spread by cross contamination through humans. Infection can be carried from house to house by footwear, clothing, and equipment as well as on the hands of the employees. Traffic between flocks—which is necessary for routine animal management practices—has been shown to be a major agent for *Campylobacter* introduction both to the environment as well as the birds themselves (Smith et. al., 2004). Shreeve and others (2000) monitored the spread of *C. jejuni* infection in a commercial multi-pen broiler house; birds were kept in confined groups and sampled sequentially. The use of individual pen drinker and feed lines as well as restricted bird-to-bird contact reduced the possibility of intraflock transmission, yet pens continued to be positive. They came to the conclusion that the farm staff, which entered the pens daily for dead collection and weighing, were the carriers of *C. jejuni* into pens (Shreeve, 2000). Biosecurity levels should not only be maintained for staff but also for visitors of all kinds, especially if they have visited or have equipment with them that has recently been to another farm. Footbaths and hand washers could reduce the level of transmission, but the proper level of biosecurity to keep flocks *Campylobacter*-negative has yet to be established (Newell and Fearnley, 2003).

Transport

Transmission of microorganisms continues from the farm into the processing plant. These microorganisms can be found in and on transportation and processing equipment, the plant environment, human employees and even the bird itself. In the bird, bacteria are present in high levels both in the gastrointestinal tract and on the feathers and skin (Musgrove et. al., 1997).

Broilers are removed from feed several hours before being taken to the processing plant. Feed withdrawal is a standard management practice that allows for the clearance of the gastrointestinal tract before slaughter to reduce the incidence of fecal material found on the carcass during processing (Keener et. al., 2004). Even when feed is withheld, there is still a chance that bacteria will make it onto the carcasses. During the withdrawal period, birds are more likely to consume contaminated litter potentially leading to increased isolation of campylobacters in the crop. Byrd and colleagues (1998) studied the effect of feed withdrawal on *Campylobacter* in the crop of market-age broilers. The results demonstrated that there was a significant increase of *Campylobacter* in crops from the start of feed withdrawal to slaughter, *Campylobacter* counts increasing further after 5 or more hours off of feed (Byrd et. al., 1998). Ceca, the blind pouches at the end of the gastrointestinal tract of birds, are known to harbor large quantities of pathogens like *Campylobacter*. Willis and others (1996) conducted a study to determine the influence of feed and water withdrawal on the detection of C. jejuni in the cloaca and ceca of market-age broilers; it was observed that *C. jejuni* detection in the cloaca increased as feed withdrawal time lengthened as well as when both feed and water were withdrawn simultaneously. After 5-12 hours of feed withdrawal, birds are loaded and transported to the processing plant. Several studies have shown that transportation crates,

if not properly cleaned and disinfected, are a source of external contamination of birds entering the processing plant (Berndtson et. al., 1996; Stern et. al., 2001; Slader et. al., 2002). It is well known that stress of handling and transporting causes disturbance of intestinal functions and increases shedding of bacteria. Stern and colleagues (1995) observed that of the 200 birds tested pre-transport, 12 % harbored *Campylobacter* at an average of $10^{2.71}$ cfu; post-transport, 56% of the chicken exteriors carried *Campylobacter* at an average of $10^{5.15}$ cfu.

Processing

In the processing plant, proper hygiene control measures take place at every step to ensure little carcass contamination. Although hygiene measures are in place, there are still numerous points along the processing line that are at higher risk for cross-contamination; these points include scalding, defeathering, evisceration, washing and chilling.

The main purpose of the scald tank is to allow easy removal of feathers during defeathering (Bell and Weaver, 2001; Mead, 2005). Birds entering the scald tank have the potential to carry high loads of fecal material on their feathers as a result of transport in crates from the farm to the plant. Stern et. al. (2001) found the scald water to be contaminated with *Campylobacter* before one of their test flocks was run, suggesting that *Campylobacter*-negative flocks can become positive if ran through the line after a positive flock. *Campylobacter* levels are generally reduced after scalding, but bacteria still in the intestine can recontaminate the carcass further in the processing line (Reiter et. al., 2005).

After carcasses have left the scalder, they move on to the highest risk areas for cross-contamination—defeathering and evisceration. The defeathering machine consists of a series of long and short rubber fingers designed to remove all the feathers on the body (Bell and Weaver, 2001). The movement of these pickers can put pressure on the carcass, releasing fecal material onto the carcass (Oosterom et. al., 1983). Berrang and others (2001) used cloacal plugs and sutures to determine if the escape of contaminated feces from the cloaca during defeathering caused an increase in *Campylobacter* numbers recovered from broiler carcasses. They found that the *Campylobacter* count was significantly larger when the cloaca was left unplugged or unsutured (Berrang et. al., 2001). During evisceration, the internal organs are mechanically removed. The rupturing and cross-contamination of the vicera is not uncommon in the plant. Oosterom et. al. (1983), Izat et. al. (1988), and Stern et. al. (2002) all found a higher incidence of *Campylobacter* on carcasses during evisceration. Since the processing machinery cannot adjust to the small variations in bird size, tearing or cutting of the intestinal tract and crop has been found to occur; damage to the viscera has been found to cross-contaminate "clean" carcasses (Rosenquist et. al., 2006). The importance of feed withdrawal is seen at this step; less material in the gastrointestinal tract leads to less material that can contaminate the carcasses. The equipment in this part of the processing line is usually not cleaned until the end of the day's operation. Bacteria that survive on these surfaces have the potential to contaminate carcasses being processed at another time (Bryan and Doyle, 1994).

Carcass washers located near the end of the processing line use chlorinated water to wash blood, tissue, and fecal matter off the carcasses. The purpose of chlorination is to prevent cross-contamination of the carcasses as well as the surrounding equipment

(Bashor et. al., 2004). Chlorine concentrations used in the washers has been found to be effective at 20 ppm (Waldroup, 1992; Bashor, et. al., 2004). The use of chemical processing aids, like organic and inorganic acids and bases, chlorine, chlorine dioxide, trisodium phosphate (TSP) and acidified sodium chlorite (ASC) have also been found to help reduce microbial populations found in and on the carcasses. These chemical processing aids have been approved by the USDA-FSIS as well as the FDA for use in decontamination of carcasses during slaughter (Capita et. al., 2002). After washing, the carcasses are moved on a conveyor belt to the chilling area.

Chilling rapidly reduces carcass temperature by means of tanks of cold water and ice, by spraying of cold water, or by the circulation of cold air (Bryan and Doyle, 1994). The USDA-FSIS requires that carcasses must be reduced to 4.4 ºC (40 ºF) within 4 hours after the evisceration step (USDA-FSIS, 1996). Many studies have shown that both cold water and cold air are effective in lowering the prevalence of *Campylobacter* on carcasses (Oosterom et. al., 1983; Allen et. al., 2000; Berrang et. al., 2001; Stern et. al., 2001; Rosenquist et. al., 2006; Figueroa et. al., 2009).

Campylobacter introduction on the farm, whether through vertical or horizontal transmission, can affect the levels of *Campylobacter* isolated from processing plant and carcasses. By reducing the risk of *Campylobacter* transmission on-farm by way of good management practices, it may be very probable to greatly decrease the microbial levels throughout all steps of processing and eventually on all finished carcasses.

Conclusion

Since their discovery in the late 1970's, *Campylobacter* has been the largest contributor to human gastroenteritis in the United States. Since poultry operations have

moved from backyard flocks into the large commercial farms of today, concerns of disease transfer to birds, either from one another or the environment has greatly increased. Identifying the vector(s) responsible for this transfer is crucial for the significant reduction of detectable levels both on the broiler farm and in the processing plant. Given that birds have constant contact with the litter from hatch until slaughter, exploration of litter as a vector for *Campylobacter* transmission and the effects of farm management practices on *Campylobacter* presence is greatly needed. While reduction of *Campylobacter* presence has already begun through the improvement of poultry housing, enhancement of general farm management practices and the application of new technology in poultry processing, there will always be room for improvement driven by the consumers need for safer poultry and poultry products.

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CHAPTER III

A ONE YEAR STUDY OF NEWLY CONSTRUCTED BROILER HOUSES FOR DETERMINING THE PREVALENCE OF *CAMPYLOBACTER*

Abstract

Campylobacter, the most frequent causative agent of bacterial gastroenteritis, is estimated to affect over 2.4 million people and cost around $1.2 billion dollars annually (Center for Disease Control. 2008). *Campylobacter* has been found on both raw poultry and poultry products. In 2009, the USDA Food Safety and Inspection Service announced the development of new pathogen reduction performance standards for *Salmonella* and *Campylobacter* both on-farm and in the poultry processing plant to reduce their prevalence on poultry products. The objective of this study was to evaluate the prevalence and distribution of *Campylobacter* within 3 newly constructed broiler houses over a year. Litter and fecal samples were collected from each house at 0, 28, and 48 d of production. Samples were serially diluted and spread onto Campy Cefex agar plates. Two 40 mL water samples were collected each production day and filtered through a 0.45 µm membrane before being placed onto Campy Cefex agar plates. All plates were flushed with a microaerophilic gas and incubated for 36 h at 42°C. Individual plates were screened for characteristic *Campylobacter* colonies and suspect colonies were confirmed using a latex agglutination kit. An additional 50 g of litter was collected during each sampling period from locations near the evaporative cooling pad, middle, and tunnel ventilation fan end to determine litter moisture content and pH. Inside and outside

temperature and humidity were collected over the entire study using a weather station. Out of 2,300 litter, 900 fecal, and 45 water samples, only 5, 6 and 1 of the collected samples were confirmed *Campylobacter* positive, respectively. Results indicated litter moisture content was different depending on location, with the tunnel ventilation fan area containing a lower level of moisture (34%) than the evaporative cooling pad (36%) and the middle (38%) ($P<0.05$). An interaction was detected for litter pH between day, location and flock. Flock 2 showed a difference between days 28 and 48 at the tunnel ventilation fan and evaporative cooling pad area. Day 0 and day 28 for flock 3 demonstrated a difference in pH between the middle and tunnel ventilation area. Litter pH equilibrated at the end of flock 3 and throughout flock 4. Temperature and humidity averaged 26.8°C and 69.3% inside and 27.6°C and 60.6% outside. In conclusion, the newly constructed houses did not show a high prevalence of *Campylobacter*. Litter moisture and humidity were at levels conducive for *Campylobacter* growth. The high litter pH and low average temperatures, along with other on-farm management strategies, may have suppressed *Campylobacter's* ability to colonize the litter.

Introduction

As of 2007, Americans on average consume 86.3 pounds of chicken, a significant increase from the 1980s when chicken consumption was on average 48.9 pounds (American Meat Institute, 2007). The major increase in poultry product consumption brings with it an increased awareness in food safety concerns for both consumers and poultry producers. *Campylobacter*, a bacterium known to be found in poultry, is the most frequent cause of bacterial gastroenteritis, also known as campylobacterosis (Newell and Fearnley., 2003). Campylobacterosis is estimated to affect over 2.4 million people and costs the United States around $1.2 billion dollars

annually. These costs are a result of physician visits, medical supplies, hospital services, and medications as well as productivity loss from poorly performed or missed work (Economic Research Service, USDA, 2000; Center for Disease Control. 2008).

Only in the last 25 years has campylobacter been recognized as an important cause of human food-borne illness. Disease control studies have demonstrated that 50% to 70% of these illnesses are attributed to consuming contaminated poultry and poultry products (Keener et. al., 2004). The manner in which poultry is raised and slaughtered leaves little possibility for the complete elimination of *Campylobacter* on broiler carcasses (Oosterom et. al., 1983; Genigeorgis et. al., 1986). On-farm, broilers may be colonized by *Campylobacter* from contaminated feed, litter, or water, and other vectors like rodents, wild birds, or humans. In the plant, raw carcasses may be contaminated by unclean machinery (carcasses pickers, neck-cutting knives, eviscerator) or by the environment (immersion chill tanks, scald tanks, employees hands and clothes). By reducing the number of bacteria on the farm, it may be possible to significantly lower the possibility for contamination in the plant.

In July of 1996, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) implemented the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems final rule with the goal of improving food safety. In this rule, FSIS established standards designed to reduce the occurrence and levels of pathogenic organisms on meat and poultry products in addition to reducing the incidences of food-borne illness associated with the consumption of those products (United States Department of Agriculture, Food Safety and Inspection Service, 1996). Also in this rule, FSIS discusses the farm-to-table strategy for the control of food safety hazards throughout the entire process of animal production. Establishing pathogen-

specific standards for both on-farm and processing facilities have allowed for the direct measurement of pathogen management and reduction. While *Salmonella* standards have been developed and enforced, *Campylobacter* standards have yet to be refined due to the lack of data available on levels of *Campylobacter* on raw poultry carcasses. FSIS announced in 2009 that they are developing new pathogen reduction performance standards for both *Salmonella* and *Campylobacter* (United States Department of Agriculture, Food Safety and Inspection Service, 2009). Therefore, the objective of this study was to investigate the prevalence of *C. jejuni* in 3 newly constructed broiler houses with the assumption that it will provide fundamental information that may be useful to government officials creating the new FSIS on-farm regulation standards for *Campylobacter* spp. in poultry.

Materials and Methods

House

A newly constructed broiler farm in Mississippi was used for sampling in this experiment. Sampling occurred in 3 of the 6 newly built houses (see Figure 3.1). The houses were solid side wall tunnel-ventilated houses, measuring 500 ft. in length and 50 ft in width. Evaporating cool pads were located on each side of the first 100 ft of the far west end of each house. Eight tunnel ventilation fans were positioned on the east end of each house. All houses contained 6 drinker lines and 3 feeder lines. The water was supplied by a well located 100 ft on the west side of all the houses on the farm. Every house was surrounded by 4 ft of grass and included a gravel drive between each for vehicle access. Rice hulls were the chosen bedding material; all houses were filled to a 5-6 cm depth with the first flock and top-dressed between every flock there-after.

Samples and Sampling Scheme

All samples were collected on day 0, 28, and 48 of a typical grow out cycle for each flock. Starting at the evaporative cool pad end, approximately 50 g of broiler litter was collected in a 2" x 2" x 2" area every 50 ft down the length of the house at 9 ft, 24 ft, and 38 ft across the house. Samples were also collected every 5 ft across the house at 100 ft, 250 ft, and 400 ft down the house (see Figure 3.2). A total of 51 litter samples per house per sampling time were collected. These particular locations were chosen from preliminary data performed prior to this study (see Appendix A). All litter samples were placed in sterile, labeled whirlpak® bags.

Fecal samples were collected from random birds at corresponding cross-points of the litter sample pattern across and down the house (see Figure 3.3). Fecal material was evacuated into sterile whirlpak® bags by expressing the cloaca. This was repeated at each cross-point (18 total), and twice more with birds chosen randomly throughout the house. Water samples from the source (the well house) and the evaporative cooling pad tanks were collected in sterile 50 ml conical tubes (Becton Dickison and Company, Franklin Lakes, NJ). The outside of the evaporative pads were dry swabbed and placed in ' Port-A-Cul' Collection and Transport Systems transport media (Becton Dickison and Company, Franklin Lakes, NJ). All samples were immediately placed on ice after collection for transport back to the laboratory.

Microbial Analysis

For litter and fecal samples, approximately 10 g and 1 g, respectively, were weighed and diluted tenfold into buffered peptone water. Samples were then stomached for 30 s at 135 rpm in a Brinkmann/Seward 400C Stomacher® (Fisher Scientific, Pittsburgh, PA). After stomaching, samples were serially diluted to 10^4 and 100 µL of

each dilution was spread, in duplicate, on Campy Cefex agar plates (Appendix B). Forty milliliters of water from both the source and evaporative cooling pad tank were filtered through 0.45 µm pore size cellulose nitrate membranes. The filters were then positioned upside down onto Campy Cefex agar plates. The evaporative cooling pad swab was removed from the transport media and directly streaked on a Campy Cefex agar plate. Using Mart anaerobic canisters and the Anoxomat Mart II system (Mart Microbiology B. V., Netherlands), all plates were flushed with a microaerphilic atmosphere (80% N_2, 10% CO_2, 5% H_2 and 5% O_2) and placed in a 20 cubic foot Precision Model 815 low temperature incubator (Thermo Scientific, Marietta, OH) at 42 °C for 36 h. At 24 h, filters from water samples were removed and plates were placed back in a microaerophilic environment for an additional 12 h before conformation. After the incubation period, plates with colonies suspected to be *C. jejuni* were confirmed using an Scimedx CAMPY (jcl)™ *C. jejuni, C. coli, C. laridis* Latex Agglutination Assay (Scimedx Corporation, Denville, NJ) (Appendix C). Plates that were confirmed positive for *Campylobacter* were set aside and counted to determine the total number of colonies on the plate.

Moisture Content

For each litter sample collected, three 15 g sub samples from each section of the house were weighed out into a tin dish to obtain an initial wet weight. Samples were then placed in a 40GC series lab oven (Quincy Lab Inc, Chicago, IL) at 105 ºC for 24 hours. Dried samples were removed from the oven and re-weighed to determine a dry weight. The moisture content (percent basis) was calculated using the following equation (American Society of Agricultural and Biological Engineers, 1998):

$$MC(\text{db percent}) = \frac{\text{Loss in weight x 100}}{\text{Weight of Dry Sample}} \quad \text{(Eq. 1)}$$

pH

Approximately 10g of litter from each location within the house was weighed and placed into a 200 mL beaker. Distilled water (100 mL) was added to the beaker and mixed for approximately 5 minutes (AOAC, 1995). An Accumet excel XL60 pH probe (Fisher Scientific, Pittsburgh, PA) was then placed in the slurry to obtain the pH reading.

Temperature and Humidity

Outside temperature and relative humidity were measured using a weather station (Onset Computer Corp., Pocassett, MA) located at the west end of the farm by the water source (the well house). A thermometer (ON-901-44008, Omega Engineering, Stamford, CT) and humidity sensor (1500LF, Americal Humirel Inc.) were used to collect data from the middle house of the three houses sampled.

Statistical Analysis

A randomized complete block design with a split-split plot over time was used to analyze litter moisture and pH data. The houses were assigned as blocks and the treatment was location within a house. Time was measured by flock and by age of the flock. The means were separated using Fishers Protected LSD. Means were considered significantly different at $P<0.05$.

Results and Discussion

From the three newly constructed broiler houses investigated over a year period, it was observed that out of 2,300 litter, 900 fecal, and 45 water samples, only 5, 6 and 1 of

the collected samples respectively were confirmed *Campylobacter* positive. Litter age may have had an effect on *Campylobacter* growth. Freshly placed litter does not have the established background microflora that used litter does. It has been found that over time the established microflora of fresh litter changes due to the addition of substances like feed, excreta, feathers, and water (Sahin et. al., 2002; Torok et. al., 2009).

Specific management practices may have had an effect on Campylobacter levels. The litter was top-dressed in each house before a new flock was placed. The placing of new, dry bedding may have changed the microbial population and reduced the possibility of cake formation in the litter during later flocks. Limited worker access to the farm may have also prevented transmission of *Campylobacter* from house to house or from the environment to the houses. It has been demonstrated that risk of flock positivity increases as the number of workers on the farm increases (Refregier-Petton et. al., 2001). The results from the litter data demonstrated that it may take *Campylobacter* longer than a year to establish itself within broiler litter. Other parameters like pH, percent moisture and climate changes may also produce an unfavorable environment for *Campylobacter* growth.

Litter moisture content was determined to increase with flock age with day 0 having a much lower litter moisture (31%) than day 28 (36%) and day 48 (41%) ($P<0.05$, Figure 3.4). It was also found that there was a very small statistical difference in average litter moisture across houses 4 (37%) and 6 (35%) ($P<0.05$, Figure 3.5). Results indicated litter moisture was also statistically different depending on location with the tunnel ventilation area containing a slightly lower level of moisture (34%) than the evaporative cooling inlets (36%) and the middle (38%) ($P<0.05$, Figure 3.6). A national survey found the ideal average moisture content for litter is around 25% (Terzich et al.,

2000). Before chick placement, the litter moisture will likely be low due to the initial heating of the house to insure proper temperatures for chicks during the first few days (Bell and Weaver, 2001). This addition of heat removes moisture from both the air and litter. As a flock accumulates body mass, it is likely that an increase in litter moisture from the release of body heat and an amplification of waste excretion will occur (see Figure 3.4). During this time, it is important to have proper ventilation to cool the birds in order to maintain feed consumption and reduce mortality (Dozier et. al. 2005). Like heating, the movement of air can also remove moisture from the litter. As air is being moved from one end of the house to another by tunnel ventilation fans, heat and moisture are transferred to the air (Czarick and Fairchild, 2003). This air is then pulled out of the house by the fans, removing heat and moisture from the air inside the house. The amount of moisture that can be removed from the litter is effected by air temperature and bird density. As air temperature increases, its ability to hold moisture increases (Ritz et. al., 2005). Higher concentrations of birds in a particular area increases the birds temperature and may not allow for the exposure of litter to the air, resulting in higher litter moisture percentages in certain areas within the house, as demonstrated in Figure 3.6 (Czarick and Fairchild, 2003).

An interaction was shown for litter pH between flock, day, and location. pH for flock 2 was statistically higher in the cooling inlet area on day 48 than the tunnel ventilation area on day 28 ($P<0.05$, Figure 3.7). The cooling inlet was also different than the other two locations on day 48 of flock 2. Flock 3 results indicated that on day 0, the pH at the tunnel ventilation area was significantly lower than the pH at the other two locations ($P< 0.05$, Figure 3.8). It was also determined that the tunnel ventilation area had a significantly lower pH on day 28 than on day 48. Litter pH on day 48 of flock 3

stabilized and this finding continued through flock 4, which had no significant changes in pH due to location and day. Typically, broiler litter ranges in pH from 9.0 to 10.0 (Blake and Hess, 2001). The optimum pH for *Campylobacter* growth is 6.5 to 7.5, but the bacteria can grow in a pH range between 4.9 to 9.0 (Doyle, 1989). In this study, the pH ranged from 8.09 to 9.30, the high end of the pH range for normal *Campylobacter* growth. High pH levels may correspond with the amount of excreta being deposited on the litter as the birds accumulate body mass. pH, as well as ammonia gas levels, increase as nitrogenous compounds—like urea and uric acid—are volatilized by microbes in the litter (Rothrock, et. al., 2008). As litter pH increases, ammonia levels increase (Blake and Hess, 2001). From day 28 to day 48 over multiple flocks, ammonia levels appeared to increase within the houses. While the pH in this study was found to have a significant interaction between flock, location and day, physiologically the small changes in pH may not have been significant enough to allow for *Campylobacter* growth.

Temperature and humidity were measured over the duration of the study and divided into seasons: spring (March, April, May), summer (June, July, August), fall (September, October, November) and winter (December, January, February). Figures 3.10, 3.11, and 3.12 illustrate the changes in average high and low temperatures as well as relative humidity, respectively. Mississippi experienced unusual amounts of precipitation during this study. Precipitation causes an increase in relative humidity. A higher outside relative humidity was observed in the fall, which happened to be the particular season that the highest level of precipitation occurred. *Campylobacter* incidence, in both broilers and humans, has been found to follow a seasonal pattern, suggesting that climatic factors may have a role in colonization (Patrick et. al., 2004; Tam et. al., 2006). Patrick et. al. (2004) observed a steep increase in *Campylobacter*

prevalence in humans and broilers at a temperature range of 8°C to 20°C. Due to the lack of *Campylobacter* presence, the possibility of climate influence or seasonality could not be observed in this study.

Insects and rodents were not huge factors on this new farm. Rodent feces, presumably mice, were found in the utility rooms attached to the houses, but were never observed. Flies were found outside the houses during the summer and fall months, but were never identified within the houses themselves. Litter beetles and worms did not appear in the litter until flock 4. The absence of these particular vectors in the houses may suggest that they did not play a role in initial *Campylobacter* transmission, but may be carriers after a farm becomes established with this bacterium.

In conclusion the newly constructed broiler houses did not show a high prevalence of *Campylobacter*. Litter moisture and humidity were at levels conducive for *Campylobacter* growth. However, the high litter pH and low temperatures, along with other on-farm management strategies, like top-dressing, water acidifying agents, and biosecurity measures, may have suppressed *Campylobacter's* ability to colonize the litter.

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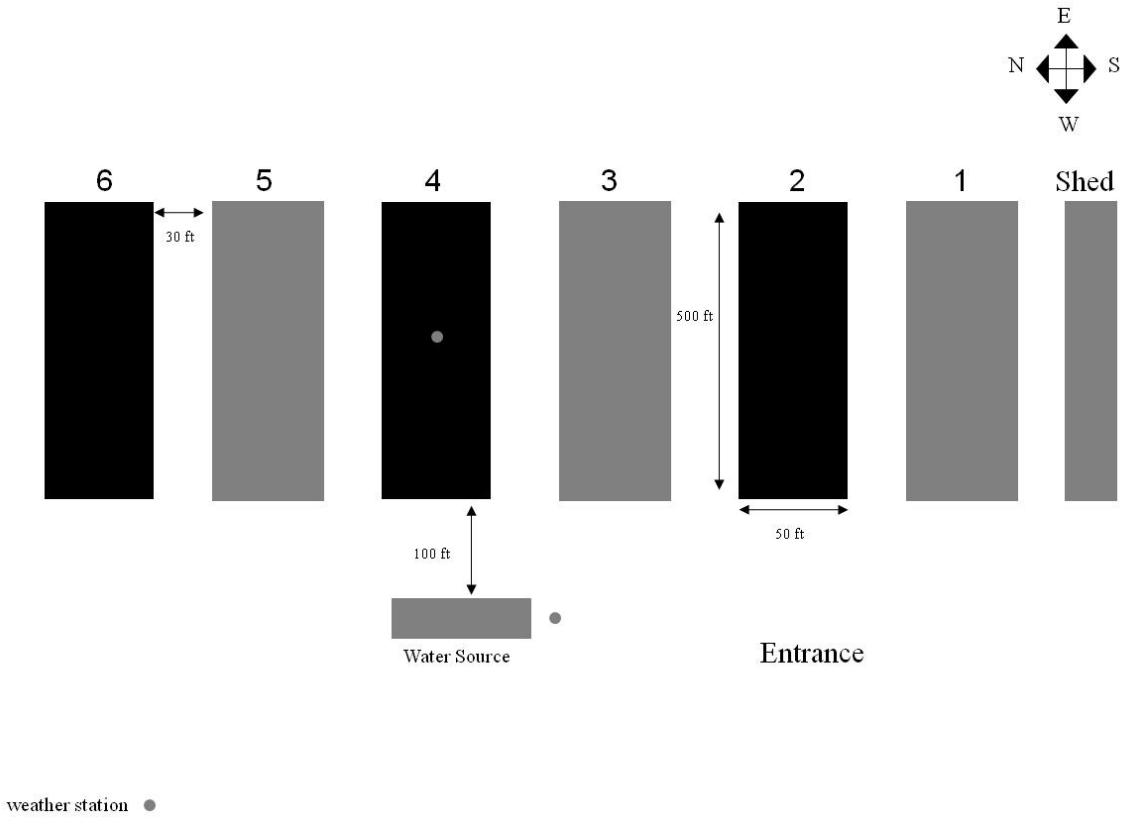

Figure 3.1 Farm schematic illustrating the spatial layout of houses 1-6 and additional buildings. Houses in black represent houses sampled for this study.

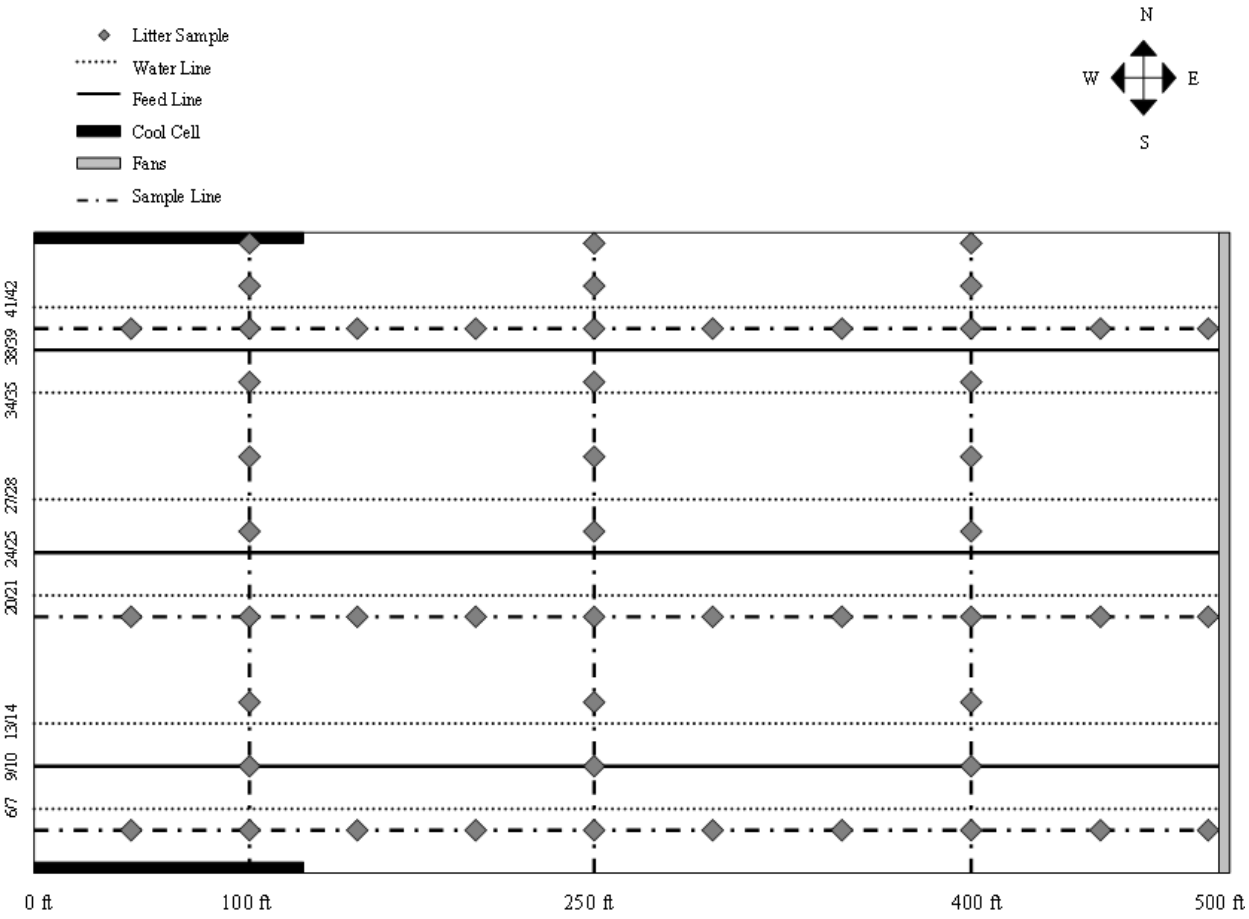

Figure 3.2 Sampling Scheme for Litter Collection

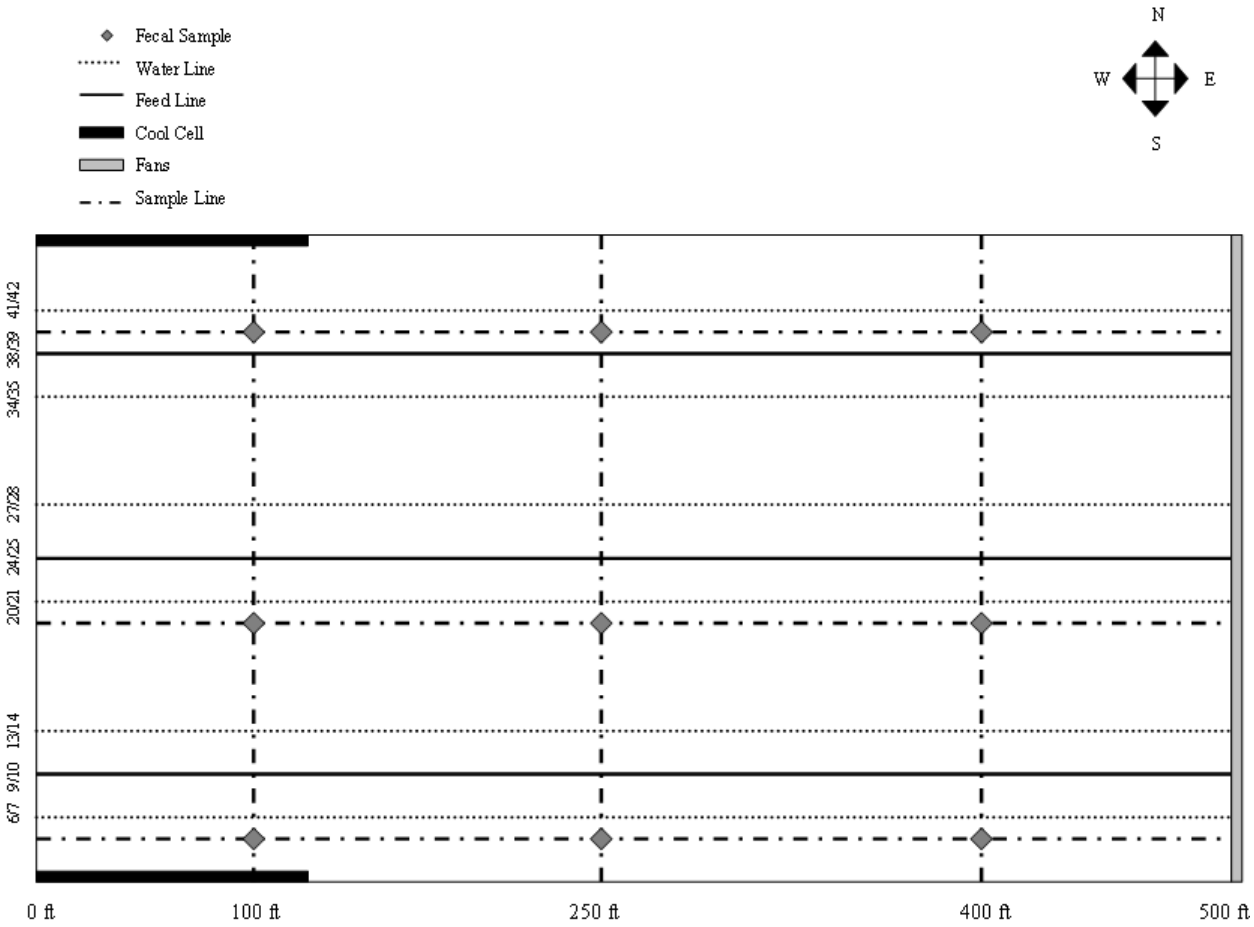

Figure 3.3 Sampling Scheme for Fecal Material Collection

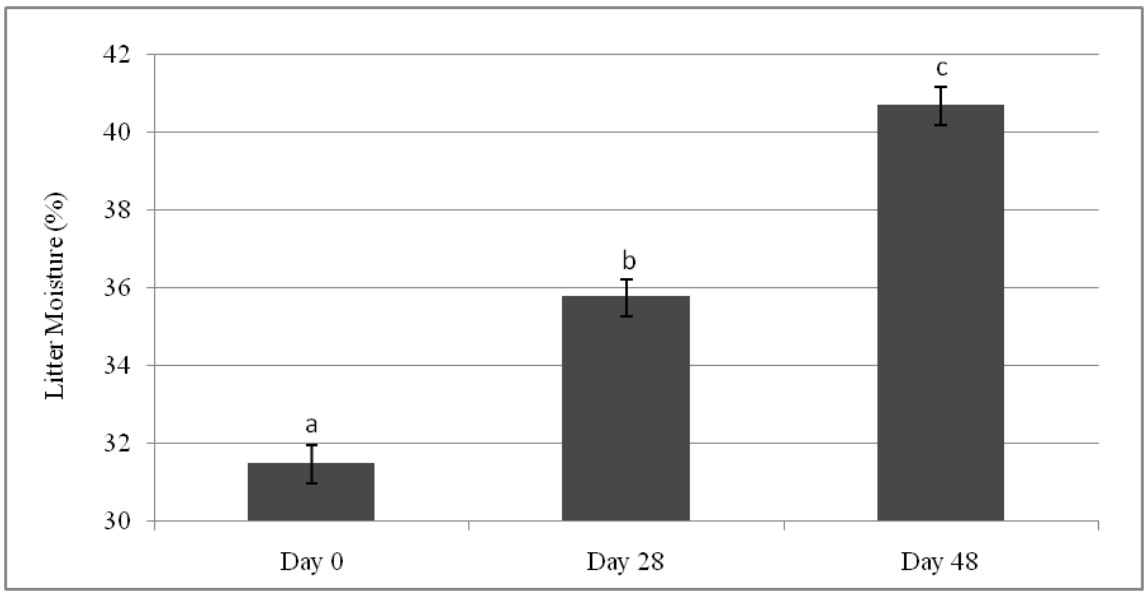

Figure 3.4 Overall average litter moisture by flock age

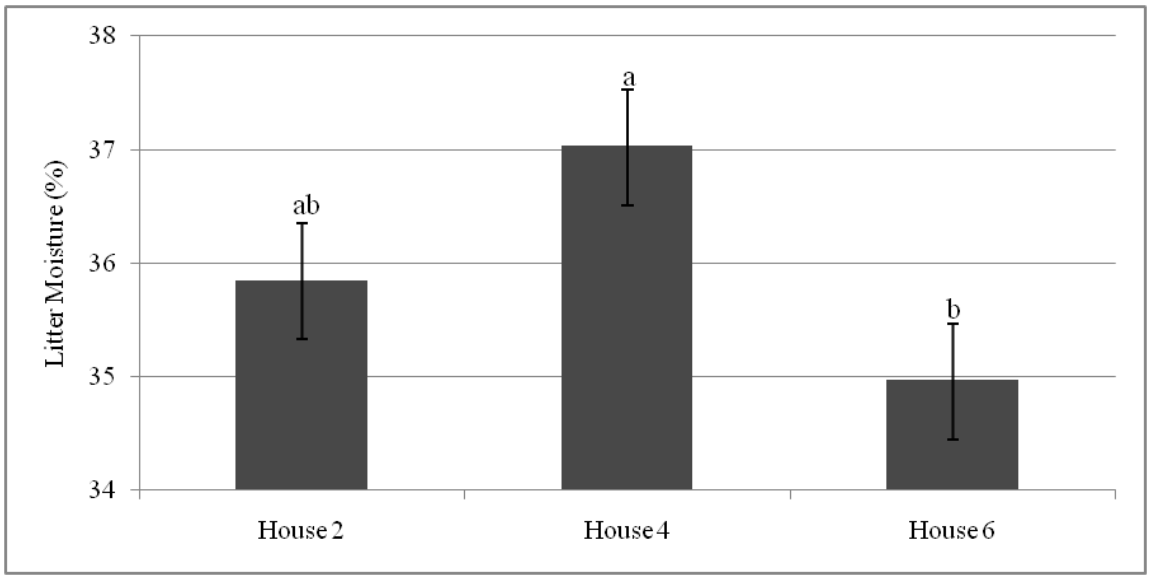

Figure 3.5 Overall average litter moisture by house

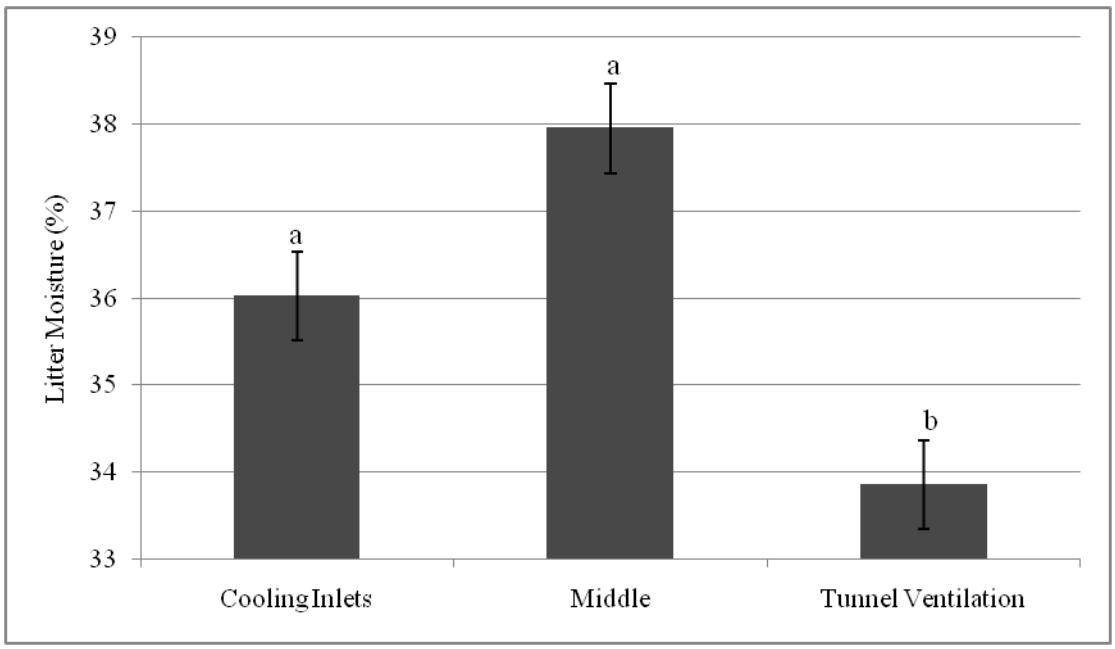

Figure 3.6 Overall average litter moisture by location within commercial broiler house

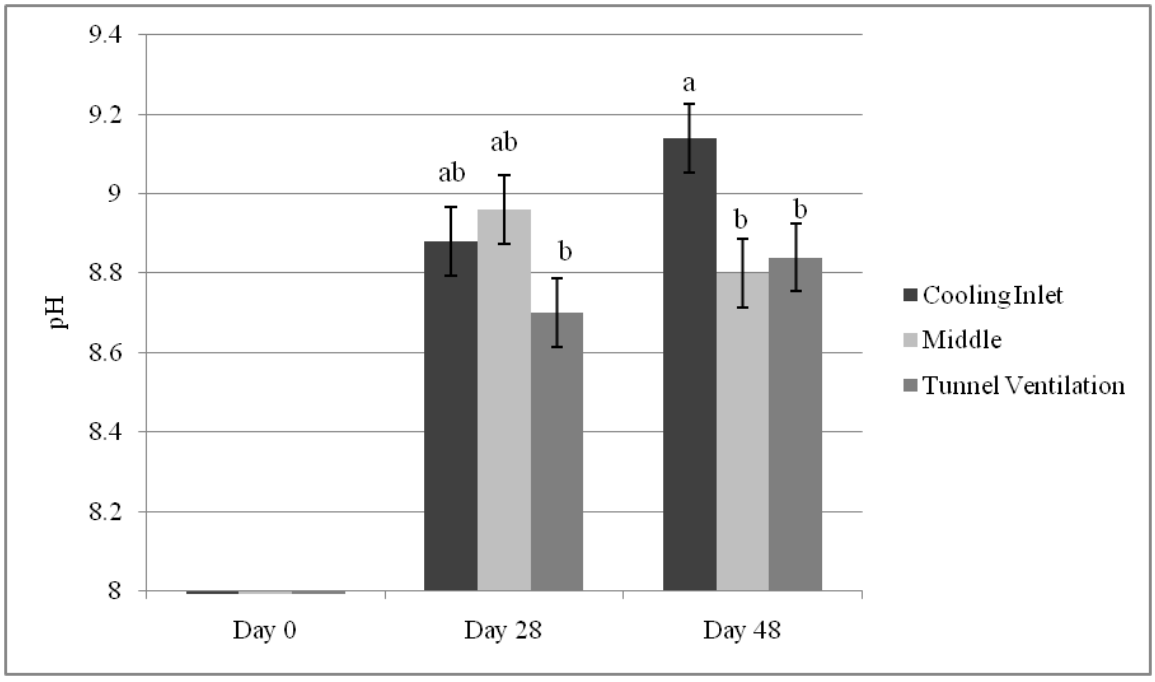

Figure 3.7 Average pH at cooling inlet, middle, and tunnel ventilation areas in a commercial broiler house for three ages during flock 2.

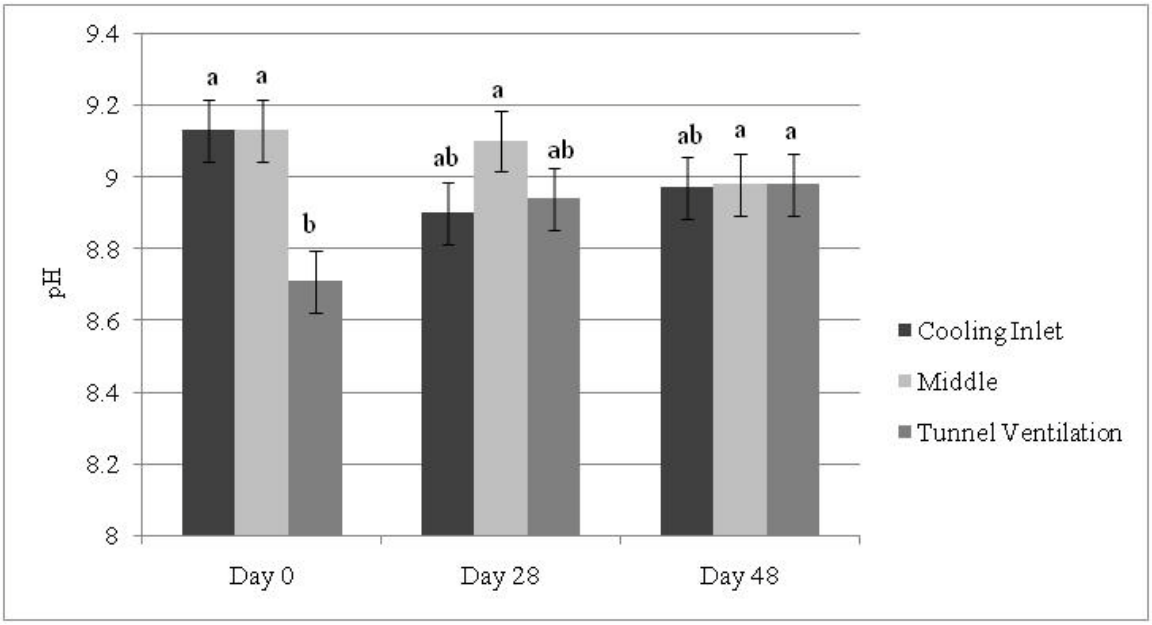

Figure 3.8 Average pH at cooling inlet, middle, and tunnel ventilation areas in a commercial broiler house for three ages for flock 3.

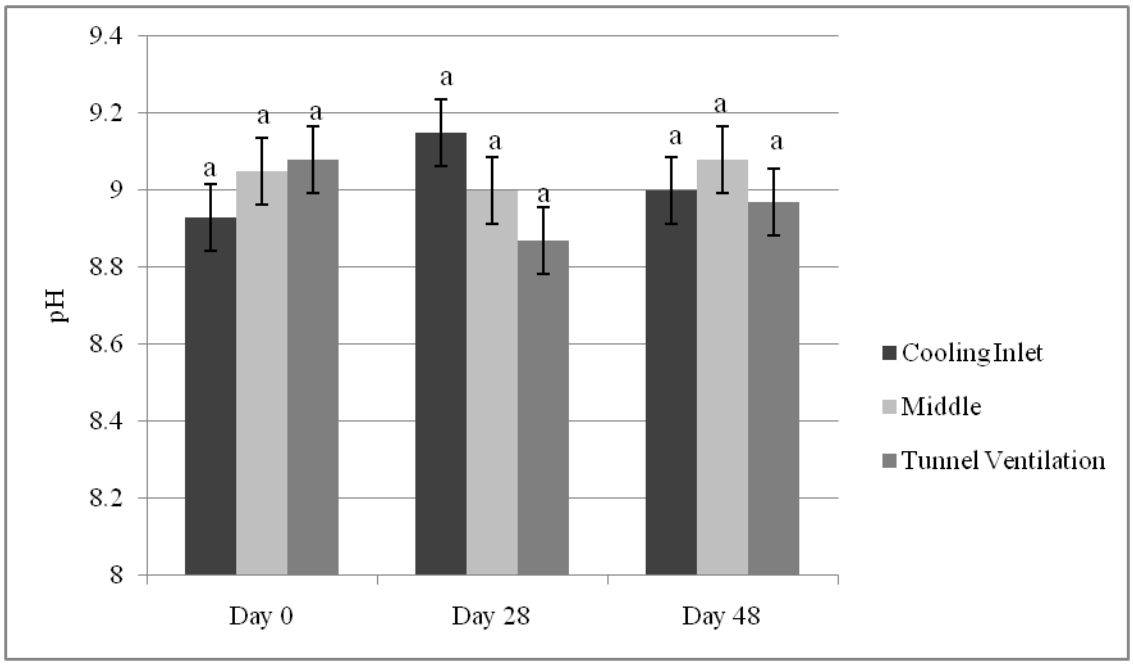

Figure 3.9 Average pH at cooling inlet, middle, and tunnel ventilation areas in a commercial broiler house for three ages during flock 4.

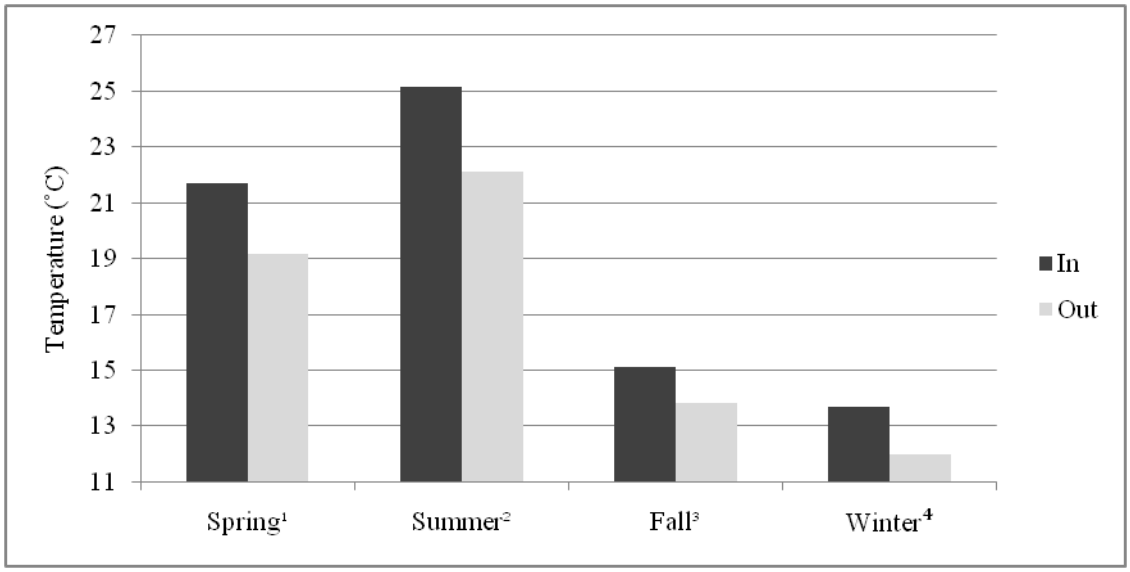

Figure 3.10 Average low temperature located inside (in) and outside (out) a commercial broiler house.

*Data not statistically analyzed
[1]- March, April, May
[2]- June, July, August
[3]- September, October, November
[4]- December, January, February

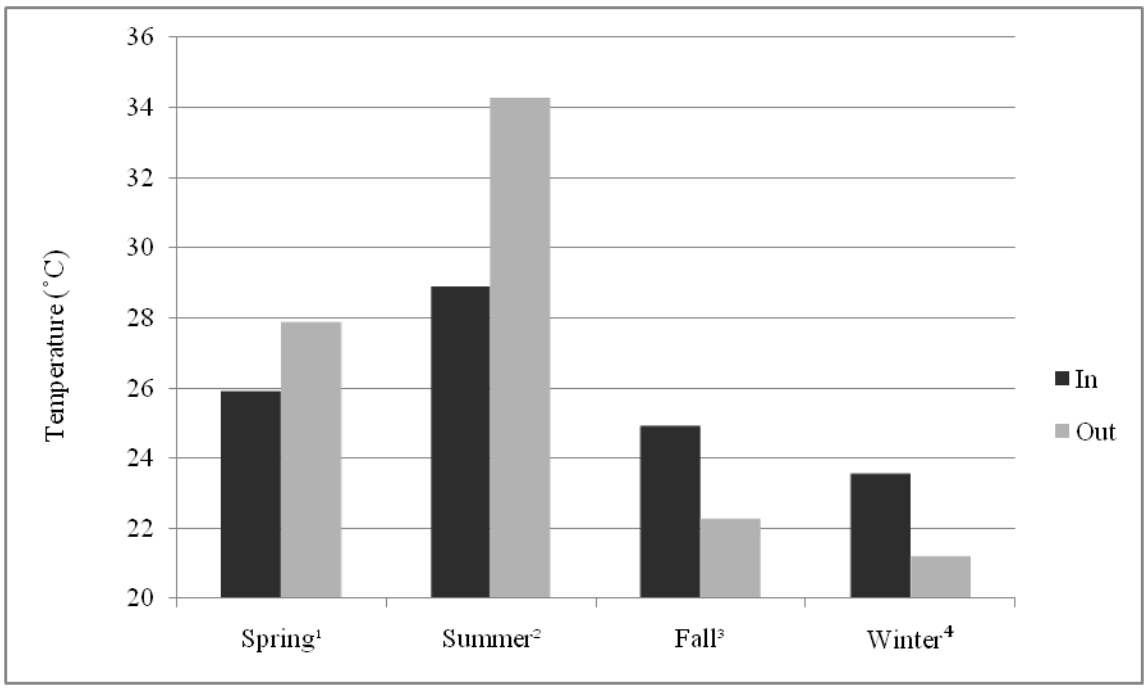

Figure 3.11 Average high temperature located inside (in) and outside (out) a commercial broiler house.

*Data not statistically analyzed
[1]- March, April, May
[2]- June, July, August
[3]- September, October, November
[4]- December, January, February

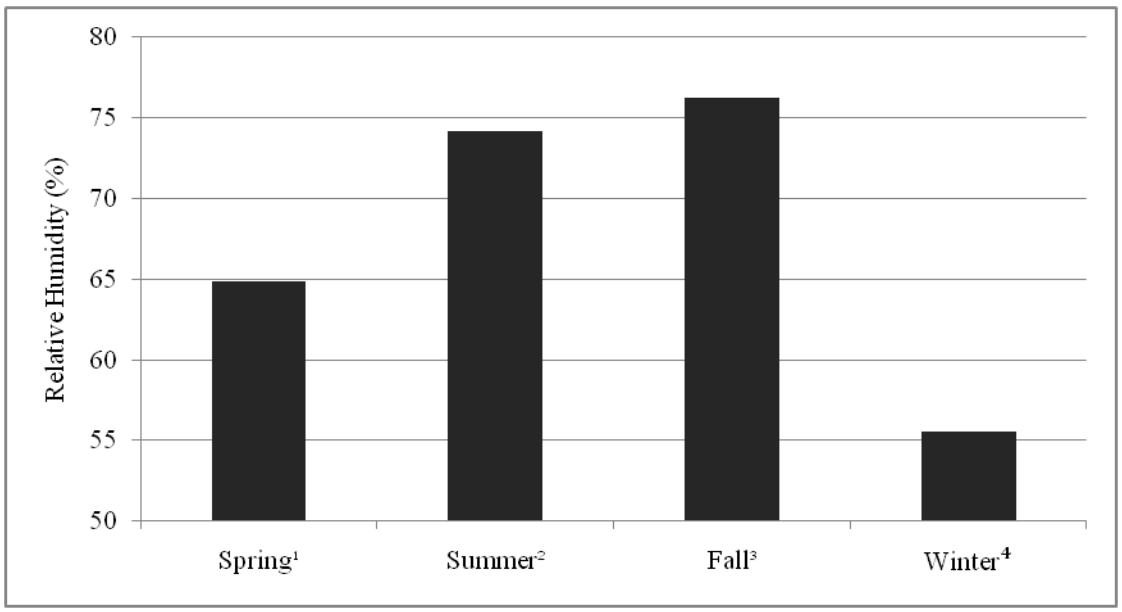

Figure 3.12 Outside relative humidity over seasons obtained from weather station located outside a commercial broiler house.

*Data not statistically analyzed
[1]- March, April, May
[2]- June, July, August
[3]- September, October, November
[4]- December, January, February

CHAPTER IV

MICROBIAL ENVIRONMENT EVALUATION

Abstract

Campylobacter spp. require a microaerophilic environment (80% N_2, 10% CO_2, 5% H_2 and 5% O_2) for growth. Several systems for creating and maintaining specific microbial atmospheres have been developed and applied since the late 1800's (Hall, 1928). The objective of this study was to evaluate *Campylobacter jejuni* growth by means of three commonly used gas delivery methods for generating a microaerophilic environment (Anoxomat, Campy Gaspak and Ziploc bags). Pure cultures *C. jejuni* cells were suspended in brucella broth and spread onto Campy Cefex agar plates. For the Anoxomat sytem, plates were positioned in a Mart anaerobic jar and flushed with a microaerphilic atmosphere using an Anoxomat Mart II system. Plates placed in Ziploc bags were flushed with a microaerophilic gas atmosphere. For the GasPak sample, plates were placed in a Mart anaerobic jar and three Campy Gaspak sachets were activated to induce the atmosphere. Plates were placed in a low temperature incubator at 42 °C for 24 hours. After 24 hours, plates were removed from incubator and counted. The entire experiment was then repeated. Results indicated no significant difference in colony counts between any of the gas delivery methods tested, but colonies grown under the Gaspak generated environment showed a smaller colony size than the other two methods. Smaller colonies when using the Campy Gaspak method could have resulted from the type of media used and the length of time plates were incubated in the incubator. In

conclusion, all three gas delivery methods were able to produce similar *Campylobacter* results between experimental runs. Initial and long term costs of equipment as well as laboratory space availability may be influential when choosing a gas delivery method for generating a microaerophilic environment.

Introduction

Campylobacter spp. require a microaerophilic environment consisting of 3-15% O_2 and 3-5% CO_2, with a few strains even able to grow under aerobic conditions containing 21% O_2 (Holt et. al., 2000). Bolton and Coates (1983) determined the 5-10% O_2 and 1-10% CO_2 was the optimum range for thermophilic campylobacters, like *Campylobacter jejuni*. Until its differentiation by Sebald and Veron in 1963, *Campylobacter* were originally part of the genus *Vibrio,* a facultative anaerobic bacteria. It was *Campylobacter*'s microaerophilic requirements and nonsaccharolytic metabolism that set them apart from *Vibrio* spp. (Dworkin, 2006). The development and application of systems for inducing and maintaining anaerobic environments have been around since the late 1800's (Hall, 1928). Little research has been conducted strictly on microaerophilic environments; because anaerobic and microaerophilic requirements are so similar, it can be assumed that the techniques and equipment used to generate an anaerobic environment can be used to induce a microaerophilic atmosphere.

Rapid atmospheric development with very low oxygen levels is crucial in the cultivation of anaerobic and microaerophilic bacteria alike (Ruangrungrote, et. al., 2008). The idea of evacuating oxygen from the environment is one of the earliest methods for inducing a specific atmosphere, first used by Pasteur and Nencki in the mid 1800's. Various forms of vacuum pumps have been used over time, including mercury and water

aspirators as well as mechanical air pumps (Hall, 1928). The passing of inert gases over media to exhaust oxygen from the environment was also used early on for creating an anaerobic atmosphere and was found to be most successful when combined with evacuation. Evacuation and the addition of gases were first performed in jar or bell-like containers where several plate cultures could be set at once. Librorius in 1886 was one of the first to combine the use of oxygen evacuation and the addition of hydrogen into jar-like container. This bell jar had rubber tube inlets and outlets to provide the atmosphere and pinchcocks to keep gases from leaking out. Botkin in 1890 included a rack to hold the dishes. The Novy jar, with its base and removable cap, was first described in 1893 and is more like the anaerobic jars used in laboratories today (Hall, 1928).

Since the development of the Novy jar, several modified containers and techniques have become available for creating both an anaerobic and microaerophilic environment. In 1964, Fletcher and Plasteridge used the techniques developed by Weiss and Spaulding (1937) to induce a particular atmosphere on microaerophilic vibrios, including *Vibrio fetus* (Fletcher and Plasteridge, 1964). The jars, which had a ten petri dish capacity, were connected to a vacuum pump and evacuated three times; after the third time the jar was filled with 10% carbon dioxide (CO_2) and 90% hydrogen (Fletcher and Plasteridge, 1964). In 1979, Karmali and Fleming tested two alternative methods—autoclave tape and Ziploc storage bags—for the growth of *Campylobacter* spp. based on the Fortner principle. The Fortner principle states that a rapidly growing facultative anaerobe reduces oxygen tension in a closed environment, allowing for the growth of other organisms with reduced oxygen tolerance. In their study, Karmali and Fleming (1979) used the facultative anaerobe *Proteus rettgeri* to reduce the oxygen tension in the atmosphere for growth of *Campylobacter*. Results showed that in using this principle,

Campylobacter was able to grow by means of both the autoclave tape and Ziploc storage bags (Karmali and Fleming, 1979). Rosenblatt and Stewart (1975) investigated the anaerobic bag and Gaspak jar method to establish an anaerobic environment. Their results demonstrated that while each method was successful at microbial propagation, there was no consistent difference in the yield of anaerobes. In 1982, Buck and colleagues evaluated and compared the effectiveness of the CampyPak II system on the isolation of *C. fetus* subsp. *jejuni* from clinical specimens. This envelope system contains a hydrogen catalyst that reduces the oxygen levels to that of a microaerophilic environment. Using this system in comparison with the routinely used evacuation jar method, they found the performance of the CampyPak II system to be identical to the jar-evacuation method, with the only difference being in price of the technique (Buck et. al., 1982). In 1984, Anoxomat, an automated jar evacuation system, was developed changing the way that anaerobic and microaerophilic environments could be created. This system automatically evacuated air from jars and replaced it using a gas mixture and pump (Braizer and Smith, 1989). This system was evaluated by Braizer and Smith (1989) who found the Anoxomat system to be fast, easy to use, and reliable. Summanen et. al. (1999) compared the growth and recovery of anaerobic bacteria in the Anoxomat system against anaerobic chambers and the GasPak system, which is similar to the CampyPak II system. These systems were all comparable to one another; each with a bacterial recovery rate above 88% of the 108 isolates tested (Summanen et. al., 1999). Sahin and others (2003) were the most recent to investigate the performance of the Anoxomat in comparison to other anaerobic systems, finding the Anoxomat provided superior growth in relation to colony size and density over the GasPak system.

The Anoxomat, GasPak system, and anaerobic bags are the three most commonly used techniques for inducing microaerophilic atmosphere in the laboratory today. With the scarce amount of research conducted to compare strictly microaerophilic environments in the laboratory, the following research will provide further insight into the best practice for creating a stable and effective microaerophilic atmosphere. The objective of this study was to evaluate the Anoxomat Mart II system, Ziploc storage bags, and the Campy GasPak system to determine if one method was more reliable and efficient in culturing *Campylobacter* than another.

Materials and Methods

Microbial Analysis

A pure *Campylobacter jejuni* culture (*C. jejuni* 700819, ATCC, Manassas, VA) stored on Cryosaver brucella beads (Hardy Diagnostics, Santa Maria, CA) was removed from a Revco Ultima -80°C Upright Freezer (Thermo Scientific, Marietta, OH) and 3 protective beads were placed into a sterile 50 ml conical tube containing 12 ml of Brucella broth. Through preliminary examination, the bead to broth ratio provided approximately 50 to 100 colony forming unit (CFU) per plate. The tube was mixed for one minute using a vortex mixer (Fisher Scientific, Pittsburgh, PA). One hundred microliters of the suspensions were spread onto 36 duplicate plates and arranged in the one of the three appropriate treatment containers for gas delivery. For the Anoxomat and Ziploc samples, plates were positioned in a Mart anaerobic jar or Ziplock Brand Double Guard Storage Bags (S. C. Johnson & Son, Inc., Racine, WI). Plates were then flushed with a microaerphilic gas mixture (80% N_2, 10% CO_2, 5% H_2 and 5% O_2) using the Anoxomat Mart II system (Mart Microbiology B. V., Netherlands) or gas directly from

the tank. For GasPak samples, plates were placed in a Mart anaerobic jar and 3 Campy Gaspak sachets (Becton Dickinson and Company, Franklin Lakes, NJ) were opened and stationed in the container. All plates were placed in a 20 cubic foot Precision Model 815 low temperature incubator (Thermo Scientific, Marietta, OH) at 42 °C for 24 hours. After 24 hours, plates were removed from the incubator and counted to determine the number of CFU present.

Statistical Analysis

Data was analyzed with a generalized complete randomized design. The means were separated using Fishers Protected LSD and were considered significant at $P<0.05$.

Results and Discussion

Three commonly used atmosphere-inducing techniques were evaluated for reliability and efficiency for the growth of *Campylobacter* spp. Results for colonies counts are presented in Figure 4.1. With 540 total plates examined for *Campylobacter* growth over all three gas delivery methods, it was determined that no significant difference was observed in mean colony counts for each method tested over all replicates (Table 4.2). *Campylobacter* colony size in the microaerophilic atmosphere generated by the GasPak EZ Campy method were much smaller than the colonies found on plates in the other methods tested. One explanation for the size difference could be the difference in time it takes the GasPak to produce a microaerophilic environment. The Anoxomat system has been found to achieve a desired atmosphere 0.5 h quicker than GasPak sachets (Summanen et. al., 1999). Colony size could have also been dependent on the length of incubation. Results from an earlier study found colonies to be too small to be measured accurately in both methods after 24 hours (Buck et. al. 1982). However, after

48 hours, colonies from the Gaspak system were found to be statistically smaller than the colonies grown in the anaerobic canisters (Buck et. al., 1982). Conversely, a later study by Summanen et. al. (1999) showed no statistical difference in the size of growth between colonies after 48 hours.

In conclusion, all three systems were able to provide a reliable microaerophilic environment for the growth of *Campylobacter* spp. When choosing a method, space and cost are two factors to consider (See Table 4.1). The initial cost of the Anoxomat system is high, but the longer term costs are relatively low (Summanen et. al., 1999). GasPak sachets are not very expensive, however they do required the same type of costly anaerobic jar that the Anoxomat system needs. Ziploc storage bags are the least expensive of the three methods. When it comes to space, plate holding capacity and incubator availability are important to account for. The anaerobic jars the Anoxomat and GasPak methods require can hold up to 36 plates while the Ziploc storage bags can only hold a maximum of 16. The Anoxomat system also uses up the most bench space; however, all three methods take up close to the same space in the incubator.

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Table 4.1 Costs for equipment and labor for each of the three microaerophilic gas delivery methods

Treatment	Equipment Cost	Labor Cost	Gas Delivery Time (mins)	Overall cost for 3,000 samples
Anoxomat Mart II System		$0.48/canister	3	
microprocessor	$9,300.00			
canister (36 plates)	$595.00			
petri dish holder	$104.00			
tank	$200.00			
total for 3,000 samples	**$19,286.00**	**$240.00**		**$19,526.00**
Campy GasPak		$0.24/canister	150	
sachet	$3.93			
canister (36 plates)	$595.00			
petri dish holder	$104.00			
total for 3,000 samples	**$15,681.00**	**$120.00**		**$15,801.00**
Ziploc Storage Bag		$0.24/bag	0.5	
bag	$0.15			
tank	$200.00			
total for 3,000 samples	**$425.00**	**$360.00**		**$785.00**

* minimum wage for July 24, 2009 is $7.25
* 3,000 samples was chosen to represent a moderately sized experiment

Table 4.2 Mean *Campylobacter* counts for three different microaerophilic gas delivery methods

Treatment	Mean Colony Count	N	SEM	P-value
Anoxomat Mat II System	72.69	179	12.17	0.87
Campy GasPak	67.01	180	12.17	0.87
Ziploc Storage bag	76.11	180	12.17	0.87

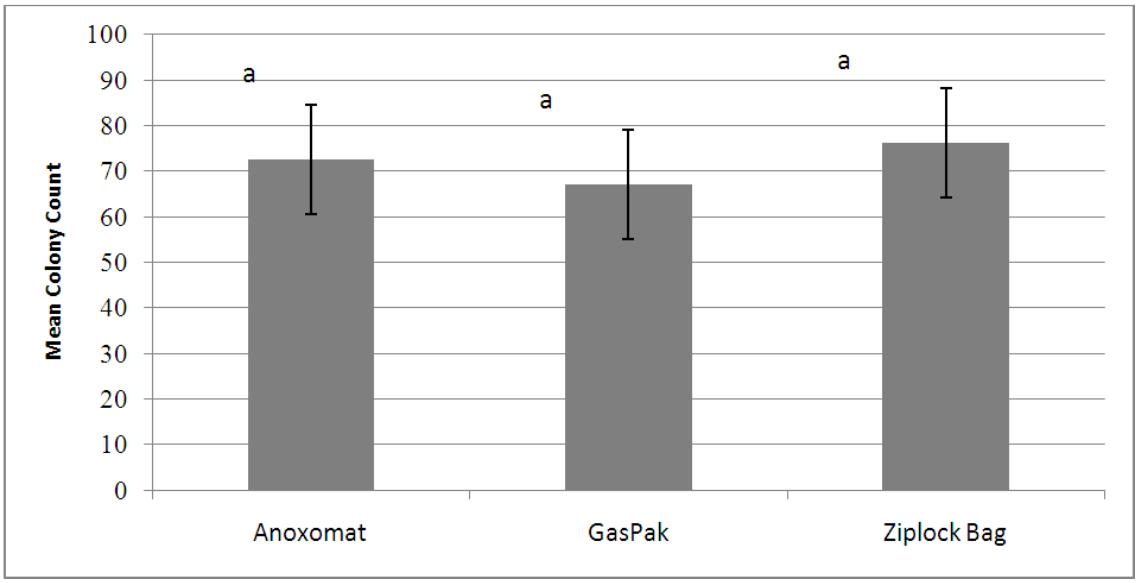

Figure 4.1 Mean Campylobacter counts

CHAPTER V

CONCLUSION

The main objective of this thesis was to investigate the prevalence of *Campylobacter jejuni* levels in newly constructed broiler houses with the purpose to provide information that may be useful in the development of on-farm *Campylobacter* standards by the USDA-FSIS. Preliminary studies were preformed in-order to develop a sampling scheme that represented different environmental regions conducive for microbial growth within a broiler house; these regions consisted of the cooling inlet end, the middle, and tunnel ventilation end. Specific sites where birds are found to congregate and create an environment adequate for pathogen proliferation were also chosen, and included the drinker lines, feeders and the wide open space between these two points. Samples were collected over an entire year to monitor for seasonal effects. Of over 3,200 litter, fecal and water samples collected for microbial analysis, less than 20 were confirmed *Campylobacter* positive. The low recovery of *Campylobacter* cells lead to the investigation of other environmental parameters including pH, moisture content, outside and inside temperature and humidity, that may have had an influence over growth. Moisture content and pH were found to be statistically different for the three regions in the houses as well as over the age of the flock. pH during the study was found to be on the high end of the pH range for *Campylobacter* growth. Average high and low temperatures were found to be below the normal *C. jejuni* tolerance of 42 ˚C. While average moisture content and humidity values were found to be conducive for

Campylobacter growth, the high pH and low temperatures may have prevented it. On-farm management practices, such as water pH treatments, top-dressing the litter, opening of houses between flocks, and competitive exclusion by other bacteria could have also prevented *Campylobacter* from becoming established in the litter.

A smaller study evaluating three gas delivery methods—Anoxomat, GasPak, and Ziploc bags—for the cultivation of *C. jejuni* were also conducted during the one year study. After testing over 180 plates for each method, no difference was found in the efficiency of providing an environment desirable for Campylobacter growth. The only difference found was in colony size, with the GasPak growing slightly smaller colonies than the other two methods.

In conclusion, *C. jejuni* was not present at detectable levels inside one year old broiler houses. Percent moisture, pH, and climate may have an effect on overall *Campylobacter* growth. This study should be continued to determine exactly when Campylobacter becomes an established part of the microflora within these newly constructed broiler houses. The other parameters measured in this study should also continue in depth to determine if and what effects they have on Campylobacter's growth once it becomes established. Only further investigation can provide the information needed to determine the need for on-farm standards in newly constructed broiler houses. Any of three of the gas delivery methods tested can successfully be used in a laboratory setting for the cultivation of *Campylobacter jejuni*. The choice of method is based on financial and laboratory space availability.

APPENDIX A

PRELIMINARY STUDY SURVEY OF CAMPYLOBACTER DISTRIBUTION IN A COMMERCIAL BROILER HOUSE TO ESTABLISH SAMPLING PROCEDURES

Abstract

Two preliminary studies were conducted to survey the distribution of *Campylobacter jejuni* within a commercial broiler house. In these studies, a commercial broiler house (43 ft wide by 400 ft long) was sampled to determine strategic locations that best represent areas conducive to *Campylobacter* growth in litter. In trial 1, samples were collected every foot across the house (43 total litter samples). In trial 2, samples were collected every 30 feet down the house at 5 different locations across the house (50 total litter samples). Locations across the house in trial 2 were determined by the results from trial 1. Fifty feet on each end of the house was excluded from the experiment. All samples were diluted in buffered peptone water, streaked onto Campy Cefex agar plates, and placed in anaerobic canisters. Canisters were flushed with a microaerphilic atmosphere (80% N_2, 10% CO_2, 5% H_2 and 5% O_2) and placed into a low temperature incubator at 42°C for 48 hours. Colonies thought to be *Campylobacter* were confirmed using a Campy-latex agglutination kit. Results from trial 1 indicated that 56% (24 out of 43 samples) of the samples collected across the house were positive for *Campylobacter* (See Figure A.1). In trial 2, 44% of the samples collected down the length of the house (22 out 50 samples) were positive for *Campylobacter* (See Figure A.2). The majority of positive samples for both across and down the house were located in the middle or tunnel ventilation fan end, with the evaporative cooling pad end having only 3 out 15 samples positive for *Campylobacter.* In conclusion, *Campylobacter* is detectable in used litter, with 1 out of every 2.3 samples collected found positive. It appears that the evaporative cooling pad end may not support the quantity of growth that was seen from the middle and tunnel ventilation fan end of the house. This information was used to develop a sampling grid for a study evaluating the prevalence of Campylobacter in the newly

constructed broiler houses of chapter 3. The grid that was designed from this study can be visualized in Figure 3.2.

Introduction

Campylobacter is one of the leading causes of diarrheal illnesses in the United States and is usually associated with the eating of raw or undercooked poultry and poultry products (Center for Disease Control, 2008). The mode by which *Campylobacter* spp. are transferred to poultry has not been clearly defined; scientists are starting to look past the processing plant to identify possible sources at a more basic level. Studies have determined litter to be a potential route of *Campylobacter* spread throughout a flock (Montrose et. al., 1984). *Campylobacter* colonization and shedding in broilers begins around week 3 of grow out (Stern et. al., 2001). Live broilers remain healthy carriers of this bacterium, which can be found in their large intestines, cecum, and cloaca in levels as high as 10^6-10^7 cfu/g of feces (Corry and Atabay, 2001). After infection, the rapid spreading of *Campylobacter* usually results in 100% colonization of a flock in a very short time (Jacob-Reitsma et. al., 1995). The objective of this experiment was to survey the distribution of *Campylobacters* on broiler litter with the intent of developing a sampling scheme to be used in a larger epidemiology study.

Materials and Methods

House

The commercial broiler house used in this preliminary study was a curtain-sided, tunnel ventilated house, measuring 43 ft wide by 400 ft long. The house contained 4 drinker lines and 3 feeder lines. Pine shavings were the preferred bedding material and were placed in the house 5 years prior to sample collection. The particular flock sampled

was on week 7 of an 8 week grow-out period. Samples were collected in November of 2008.

Sampling Scheme

In trial 1, approximately 50 g of litter was collected every foot across the house, for a total of 43 litter samples. In trial 2, approximately 50 g of litter was collected every 30 feet down the house at 5 different locations across the house, totaling 50 total litter samples. All litter samples were placed in sterile, labeled whirlpak® bags and were immediately placed on ice for transport back to the laboratory.

Microbial Analysis

For litter samples, approximately 10 g was weighed and diluted tenfold into buffered peptone water. Samples were then stomached for 30 s at 135 rpm in a Brinkmann/Seward 400C Stomacher® (Fisher Scientific, Pittsburgh, PA). After stomaching, samples were serially diluted to 10^4 and 100 µL of each dilution was spread, in duplicate, on Campy Cefex agar plates. Plates were then placed in Mart anaerobic canisters (Mart Microbiology B. V., Netherlands). Using an Anoxomat Mart II system (Mart Microbiology B. V., Netherlands), all plates were flushed with a microaerphilic atmosphere (80% N_2, 10% CO_2, 5% H_2 and 5% O_2) and placed in a 20 cubic foot Precision Model 815 low temperature incubator (Thermo Scientific, Marietta, OH) at 42 °C for 48 hours. After the incubation period, plates with colonies suspected to be *C. jejuni* were confirmed using a Campy-latex agglutination kit by Panbio (Panbio, Inc. 9075 Guilford Rd. Columbia, MD 21046).

Results and Discussion

No statistical analysis was run for this data. Results indicated that 56% (24 out of 43 samples) of the samples collected across the house width in trial 1 were positive for *Campylobacter* (See Figure A.1). Forty four percent of the samples collected down the house length (22 out 50 samples) in trial 2 were positive for *Campylobacter* (See Figure A.2). The majority of positive samples were located in the middle or tunnel ventilation fan end of the house. The evaporative cooling pad end of the house only had 3 out 15 samples positive for *Campylobacter* indicating that the environment may have been to harsh to support growth during our sampling period.

In conclusion, *Campylobacter* seems to be present in reused broiler litter, with 1 out of every 2.3 samples collected positive for *Campylobacter*. It appears that the cooling pad end may not support the quantity of growth that was seen from the middle and fan end of the house. This information was used to develop a sampling grid for a study that evaluated the prevalence of Campylobacter in newly constructed broiler houses. The grid designed from this study can be visualized in Figure 3.2.

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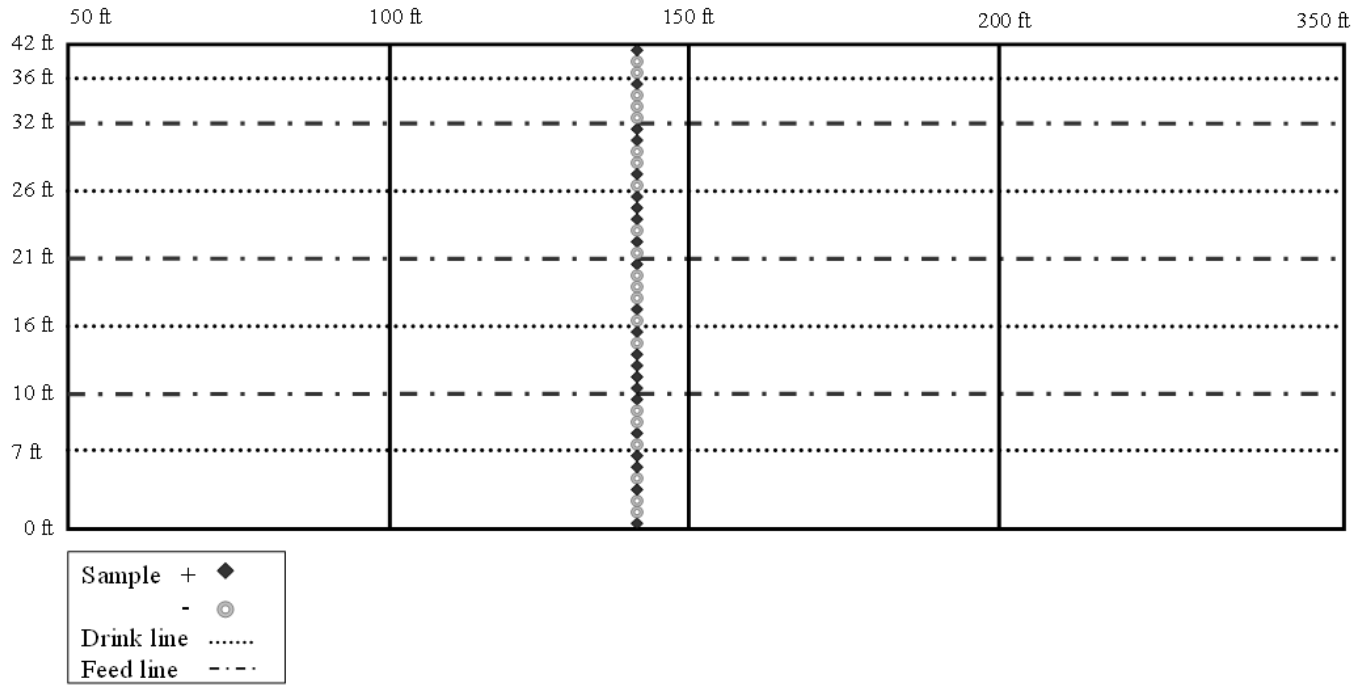

Figure A.1 Sample locations confirmed Campylobacter positive across the width of a commercial broiler house

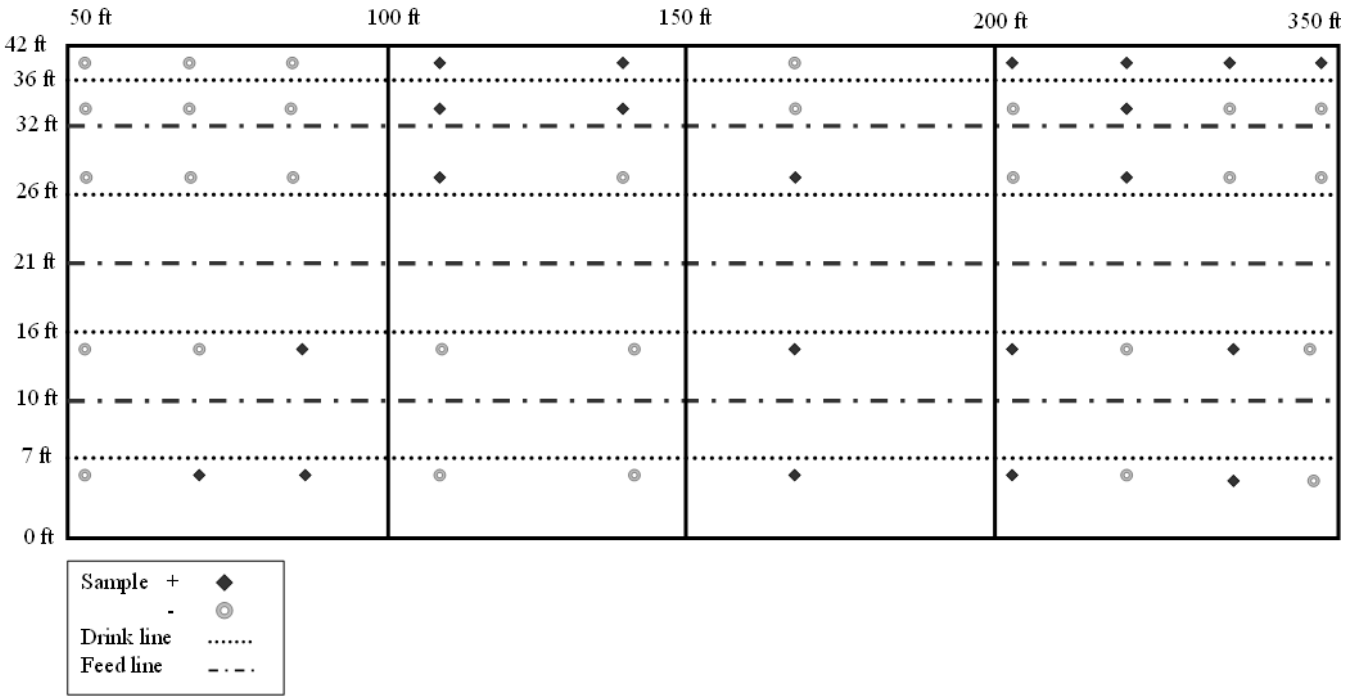

Figure A.2 Sample locations confirmed *Campylobacter* positive across the length of a commercial broiler house

APPENDIX B

CAMPY-CEFEX AGAR

ACUMEDIA™ CAMPY-CEFEX AGAR
CAT. # 7718

FORMULA

Enzymatic Digest of Casein	10.0 g
Enzymatic Digest of Animal Tissue	10.0 g
Sodium Chloride	5.0 g
Yeast Extract	2.0 g
Dextrose	1.0 g
Sodium Pyruvate	0.5 g
Ferrous Sulfate	0.5 g
Sodium Bisulfite	0.3 g
Cycloheximide	0.2 g
Agar	15.0 g

Final pH: 7.0 ± 0.2 at 25°C

SUPPLEMENTS
Cefoperazone, 0.033 g
Sterile laked horse blood, 5%

SUBSTITUTION
Sterile lysed horse blood, 5% for sterile laked horse blood, 5%

DIRECTIONS
Dissolve 44.4 grams of the medium in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121 ˚C for 15 minutes. Cool to 50 ˚C and aseptically add 10 ml of filter sterilized solution containing 0.033 g of Cefoperazone and 5% of sterile laked horse blood.

APPENDIX C

BUFFERED PEPTONE WATER

DIFCO™ BUFFERED PEPTONE WATER
CAT. # 218105

FORMULA

Peptone	10.0 g
Sodium Chloride	5.0 g
Monoptoassium Phosphate	3.5 g

Final pH: 7.0 ± 0.2

DIRECTIONS

Dissolve 20.0g of the powder in 1 L of purified water. Mix thoroughly. Autoclave at 121 ˚C for 15 minutes

APPENDIX D

SCIMEDX®-CAMPY(JCL)™ CULTURE CONFORMATION TEST FOR

CAMPYLOBACTER JEJUNI, C. COLI AND *C. LARIDIS*

SCIMEDX®-CAMPY(JCL)™ CULTURE CONFORMATION TEST FOR
CAMPYLOBACTER JEJUNI, C. COLI AND C. LARIDIS
CATALOG # L-CAM01T

MATERIALS

Scimedx®-campy (jcl)™ **Latex Detection Reagent** (2 x 3.5 mL)-consists of rabbit antiserum to common antigens of selected Campylobacter species bound to latex particles suspended in a buffer containing a preservative.

Scimedx®-campy (jcl)™ **Extraction Reagent** (2.8 mL)- consists of a dilute solution of hydrochloric acid

Scimedx®-campy (jcl)™ **Neutralization Reagent** (2.8 ml)- consists of glycine buffer containing a preservative

Scimedx®-campy (jcl)™ **Positive Antigen Control Reagent** (2.7 ml)- consists of neutralized acid extract of appropriate Campylobacter organisms in buffer containing a preservative.

Test Slide

Applicator sticks

High intensity lamp

Slide Rotator

PROCEDURE

1. Remove reagents from refrigerator and allow warming to room temperature before use.
2. Label one circle on the test slide for each specimen to be tested.
3. Identify one circle for the positive control and another for the negative control reactions.
4. Remove the cap and tip protector from the vial of Extraction Reagent. While holding the vial vertical position, dispense one free-falling drop of Extraction Reagent into each specimen circle and the negative control circle. Replace the tip protector and cap.
5. Touch one isolated colony with the end of a wooden applicator stick to remove it from the agar surface.Generally, one colony with a diameter of 2mm (about the diameter of the applicator stick) will provide an adequate inoculum. If colonies are small, yet distinct from the surface of the agar, it may be necessary to pick 2-6 colonies. However, care must be taken as too much inoculum may contribute to poor readability.

6. Make homogenous suspension by rotating the inoculum containing stick in the Extraction Reagent within the appropriate specimen circle. It is very important to dissociate all visible clumps of the inoculums and distribute the suspension over the entire area within the circle. Repeat steps 5 and 6 for each specimen to be tested. No incubation time is required for this step. Proceed to step 7.
7. Remove the cap and tip protector from the vial of Neutralization Reagent. While holding the vial in a vertical position, dispense one free-falling drop of Neutralization Reagent into the fluid spread in each specimen circle and the negative control circle. Replace tip protector and cap.
8. Remove the cap from the Positive Control Reagent and wipe the tip with a clean-lint free tissue. While holding the vial in a vertical position, dispense one free falling drop into the positive control circle. Replace the cap.
9. Gently re-suspend the Latex Detection Reagent to assure a homogenous suspension. Do not shake the reagent and avoid the formation of foam or bubbles.
10. Remove the cap from the Latex Detection Reagent and wipe the tip with a clean lint-free tissue. While holding the vial in a vertical position, dispense one free-falling drop Latex Detection Reagent into each circle, as appropriate, on the slide. Avoid forming bubbles on the dropper tip as the latex reagent is dispensed. Do not touch the tip of the dropper vial to the material on the slide. Replace the cap.
11. At this point each circle with have received the following:

Specimen: 1. Extraction Reagent, 2. Bacterial colony(ies), 3. Neutralization Reagent and 4. Latex Detection Reagent

Negative control: 1.Extraction Reagent, 2. Neutralization Reagent and 3. Latex Reagent

Positive control: 1. Positive control Reagent and 2. Latex Detection Reagent

12. Use a separate applicator stick to mix the contents of each circle thoroughly.
13. Place slide on a rotator and rotate at 100-110 rmp for 5 minutes at room temperature.
14. After rotation is completed, immediate observe the reactions for visible agglutination under a high intensity light.
15. A positive test is indicated when the Latex Detection Reagent clearly agglutinates with the test specimen and no agglutination occurs in the negative control circle. The presence of agglutination in the negative control circle renders the test invalid.
16. A negative test is indicated by the absence of agglutination of the Latex Detection Reagent with the test specimen.

Scimdex Corporation Denville, NJ 07834 USA.

APPENDIX E

MOISTURE CONTENT PROTOCOL

MOISTURE CONTENT PROTOCOL

MATERIALS

Aluminum weighing tin

Balanced scale

Drying oven, at 105 ˚C

PROCEDURE

1. Place weighing tin on balanced scale and weigh. Tare scale.
2. Place a sample of about 15 g of litter in weighing tin and weigh. Repeat procedure twice more.
3. Place the weighing tins in a drying oven set at 105 °C
4. After drying for 24 hours, remove the weighing tins from the oven and allow to cool.
5. Weigh the weighing tins with litter (W3).
6. Use equation to calculate percent moisture content of litter sample.

CALCULATIONS

Calculate the moisture content of the soil as a percentage of the dry soil weight.

$$MC(\text{db percent}) = \frac{\text{Loss in weight} \times 100}{\text{Weight of Dry Sample}}$$

REFERENCE

American Society of Agricultural and Biological Engineers. 1998. ASAE S358.2 Moisture Measurement-Forages. St. Joseph's, MI.

APPENDIX F

PH PROTOCOL

PH PROTOCOL

MATERIALS
Weight boat
Balanced scale
200 mL beaker
dI water
graduated cylinder
pH meter

PROCEDURE

1. Calibrate pH meter using standardized calibration solutions (pH 4.0, 7.0, and 10.0), rinsing probe before and after with dI water..
2. Place weight boat on balanced scale and weigh.
3. Place 10 g of litter into weight boat and weigh. .
2. Place litter in clean 200 ml beaker and add 100 ml of dI water to beaker.
3. Allow mixture to sit for 5minutes.
4. Using probe, take pH from beaker and record.
5. Rinse probe with dI water

REFERENCE

AOAC 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Arlington, VA

APPENDIX G

WATER ANALYSIS PROTOCOL

WATER ANALYSIS PROTOCOL

MATERIALS

0.45 nm nitrocellulose membrane filter funnel

funnel adapter

flask

funnel stopper

tubing

vacuum system

forceps

50 mL pipet

PROCEDURE

1. Invert water sample several times to mix contents.
2. Using 50 mL pipet, take 40 ml water from sample. Place water into vacuum filtration system, allowing water to flow through filter into the flask.
3. With sterile forceps, remove filter straight from funnel and invert it onto media.
4. Place media into chambers and induce a microaerophilic environment.
5. Place chambers into 42 ˚C incubator. After 24 hours, remove filter from media with sterile forceps.
6. After re-inducing a microaerophilic environment, place media back into incubator for another 12 hours.