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# The Effect of Used Broiler Litter on the Growth and Persistence of Campylobacter

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# THE EFFECT OF USED BROILER LITTER ON THE GROWTH AND PERSISTENCE OF *CAMPYLOBACTER*

By

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PERSISTENCE OF CAMPYLOBACTER

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Litter is a possible source of *Campylobacter* colonization for broilers as well as contamination of crops when used as fertilizer. A survey of Arkansas broiler litter indicated that *Campylobacter* recovery rates were higher in pine shavings and rice hulls than sand. Two experiments utilized three types of litter, which were artificially contaminated with *Campylobacter*. After 24 hours no *Campylobacter* could be recovered from any sample. *Campylobacter* growth was also examined for used pine shaving litter in varying conditions: aerobic atmosphere, micro aerobic atmosphere (6% $O_2$), and moisture content. *Campylobacter* was recovered for all treatments at the initial sampling, and by the 12 hour sampling time, only the added moisture and micro aerobic atmosphere yielded recoverable *Campylobacter*. This research suggests that without birds present in the house to shed fresh *Campylobacter* cells onto the litter, that the litter itself is incapable of harboring the bacteria long enough to colonize sequential flocks.

## DEDICATION

I would like to dedicate this thesis to my parents Clay and Gail Williams, without their help I could not have finished this, and to my brother Matt, for his words of encouragement.

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# CHAPTER ONE

# INTRODUCTION

*Campylobacter* is the leading cause of food borne illness in the United States and other developing countries (CDC 2008). One of the major reservoirs of this pathogen is poultry meat and poultry meat products. Broilers through a symbiotic relationship with *Campylobacter* are initially colonized, and through current processing techniques the meat can become contaminated. As of now, no solid evidence exists to show the primary route of flock colonization. Many theories exist, and have been investigated such as pests (Ekdahl et al., 2005), farm workers (Berndtson et al., 1996), water (Pearson et al., 1993), farm animals (Gregory et al., 1997) passage from primary breeders (Cox et al.,2002) and litter (Montrose et al., 1984) as a source of colonization.

Used broiler litter has been shown to aid in the colonization and spread of *Campylobacter* in poultry flocks (Montrose et al., 1984; Willis et al., 2002; Line, 2002; Pope et al., 2000; Payne et al., 1999; Hutchinson et al., 2004). *Campylobacter* isolated from litter has been shown to be resistant to several antibiotics such as ampicilin, penicillin, tetracycline and others (Kelley et al., 1998). This resistance can be very dangerous if it becomes widespread among *Campylobacter* spp., as means of treatment would be reduced or ineffective.

Broiler litter is produced at an average of 0.71 – 1.73 tons/1000 birds (Patterson et al., 1998). According to Chamblee et al. (2002), in Mississippi alone there were 739.9 million broilers produced in the year 2000 which would produce 1.2 million tons of litter per year at a rate of 1.6 tons/1000 birds. Broiler litter is used for crop fertilizer (Nicholson et al., 2005), cattle feed (Cross et al., 1978; Jeffrey et al., 1998) and reused in broiler houses for sequential flocks (Chamblee et al., 2002). Because of this reuse, knowledge is needed to show how pathogens such as *Campylobacter* can survive in used broiler litter, so that litter can be properly handled to reduce pathogen numbers.

The purpose of this research is to determine if *Campylobacter* will grow and persist in used broiler litter. The first experiment was a survey of litter from commercial broiler houses in northern Arkansas, for the presence of *Campylobacter*. In the second experiment, 25 grams of litter was artificially inoculated with *Campylobacter* to determine if, current recovery methods could be used to detect the organism in litter over time. The third experiment used a larger quantity of litter, 4 1/2 inches deep, which was tested for *Campylobacter* over time, to determine if the type of litter had an effect the bacterium's ability to persist in the litter. In the fourth experiment, litter was inoculated with *Campylobacter* and tested over time to determine if, atmosphere and/or moisture had an effect on the persistence of *Campylobacter* overtime.

If *Campylobacter* can not survive in these conditions, then it is highly unlikely that it will survive, in broiler litter. Indicating that the colonization of new broiler flocks by used litter is also highly unlikely. If *Campylobacter* is shown to survive in litter, then steps are needed to reduce *Campylobacter* numbers in litter. These steps will hopefully provide a safer end product for the consumer.

# CHAPTER TWO

# LITERATURE REVIEW

## Introduction

Bacterial food borne illnesses are a major concern not only for meat producers and processors but also for the general public. These bacteria are not a problem with adequately cooked meat however; undercooked meat can lead to serious, sometimes-fatal illnesses (Mead et al., 1999). Even though adequate cooking will eliminate harmful organisms, it is up to the meat producing industry to try and reduce most, if not all, of the possible pathogenic bacteria from meat to ensure a safe product for public consumption. *Campylobacter*, while not the high profile bacteria that *E. coli* and *Salmonella* spp. are, it is a major concern to the meat and especially the poultry industry. In poultry, many routes of flock colonization have been researched, but, as of yet, no definitive route has been found. One possible route of flock colonization is litter, due to the constant contact birds have with litter. As a result of this contact, litter appears to be a viable pathway for colonization from one bird to the next as well as from one flock to the next successive flock. Several research experiments have been conducted to support this theory that birds are able to contaminate litter with *Campylobacter* which leads colonization of other birds present (Montrose et al.,

1984; Willis et al., 2002; Line, 2002; Pope et al., 2000; Payne et al., 1999; Hutchinson et al., 2004).

### *Campylobacter* spp.

*Campylobacter* is characterized as a gram negative, curved rod or spiral in shape, with one or more turns; it is motile with a single polar flagellum. Campylobacter is non-sporeforming, but can form coccoid bodies, energy is obtained from amino acids or tricarboxylic acid cycle intermediates (Holt et al., 2000). The optimal growing conditions for *Campylobacter* include a micro aerobic atmosphere (6% oxygen, 10% carbon dioxide and 84% nitrogen) and an incubation temperature of $42^{O}$ C (Kiggins et al., 1956). This incubation temperature is higher than that of many other bacteria, but *Campylobacter* is capable of growing in a wide range of temperatures and environmental conditions, even surviving liquid nitrogen storage (Gorman et al., 2004).

Because of the increasing occurrence of this organism as a human pathogen in the 1970's, starting in 1982 the CDC started a national surveillance program for *Campylobacter*. Through this program a selective medium was developed which allowed for more routine testing for *Campylobacter* in patients (CDC 2008, George et al., 1978). This medium enhanced the growth of *Campylobacter fetus* and other aero tolerant strains, which consisted of Brucella agar enhanced with iron sulfate, sodium metabisulfite and sodium pyruvate. The agar along with a micro aerobic environment allowed for the growth of *Campylobacter* 1-2 days earlier than unsupplemented Brucella agar (George et

al., 1978). In 1999, a patent was issued for *Campylobacter* Cefex agar to N.J. Stern. This agar was similar to the previously described agar, except it contained iron sulfate, pyruvic acid, sodium bisulfite, Brucella agar and lysed horse blood. The agar also included two antibacterials, cyclohexamide and, ceferapazone making the media more selective for *Campylobacter*.

**Impact on Society**

Campylobacteriosis, causes mild to severe gastroenteritis in humans, typically lasting for 2-7 days with a 2-10 day incubation period (Cox et al., 1987). Symptoms include non-bloody diarrhea, fever, abdominal pain and cramps (Cox et al., 1987). The Centers for Disease Control report that most cases do not require special medical treatment, but in severe cases the patient is treated with erythromycin or fluoroquinolone type antibiotics (CDC 2008). The disease is rarely fatal but has been linked to a potentially deadly disease called Guillaine-Barre syndrome, which affects the central nervous system slowly paralyzing the victim. An estimated 40% of these cases started with *Campylobacter* infections (Kaldor et al., 1984, Buzby et al., 1997).

The United States Department of Agriculture has estimated the cost of major food borne pathogens: *Campylobacter*, *Escherichia coli*, *Salmonella,* and *Listeria monocytogenes* at $6.9 billion annually with *Campylobacter* accounting for $1.2 billion alone (USDA ERS, 2005). This includes lost wages, hospital bills and deaths; however, this total does not include money lost by the meat industry (USDA ERS, 2005). There are an estimated 2.5 million cases of

Campylobacteriosis in the United States per year with 80% being food borne (Altekruse et al., 1999; Mead et al., 1999; Sahin et al., 2002). *Campylobacter* accounts for 14% of all food borne illness, 17.3% of hospitalizations and 5.5% of deaths, approximately 124 annually, these startling statistics make *Campylobacter* one of if not the leading cause of human food borne illness (Mead et al., 1999; CDC, 2008).

According to Denis et al. (2001), approximately 75% of poultry products in store display cases are contaminated with *Campylobacter*. Another study of 46 broiler chickens found that 83% were contaminated with *Campylobacter,* and there chickens were positive for *Campylobacter* for at least 96 hours at $4^{O}$ C (Grant et al., 1980). Because *Campylobacter* can survive in a broad range of temperatures, it is extremely important that meat producers and especially poultry producers reduce the numbers on products before these products enter the food market. Even though many steps and precautions (HACCP, farm biosecurity) have been taken to control and reduce the incidence of *Campylobacter*, it is still frequently found in the food supply. The steps that have been taken to control *Campylobacter* occur not only in the processing plant but on the farms and in the hatchery (Pattison et al., 2001; Newell et al., 2003; Giessen et al., 1992, Cox et al., 2002).

**Modes of Transmission**

Horizontal transmission, from one bird to another bird in the same flock or house, is thought to be the most probable source of colonization (Sahin et al.,

2002). This type of transmission can also be caused by other farm animals (Gregory, et al., 1997), employees (Berndtson,. et al., 1996) and even from one flock to the next flock in the same house (Sahin et al., 2002). *Campylobacter* is thought to be spread by pests such as litter beetles, but Skov et al., (2004) reported that although *Campylobacter* was isolated from litter beetles, beetles were only *Campylobacter* positive while broilers where present. *Campylobacter* from successive flocks in the same houses have been shown to be clones of each other, indicating horizontal transmission of *Campylobacter* from one flock to the next (Petersen et al., 2001).

Vertical transmission, from parents to offspring, is also a possible source for the spread of the *Campylobacter*. As with most broiler flocks, many broiler breeder flocks are colonized with *Campylobacter*. During mating when a rooster ejaculates, the sperm can come into contact with feces. As a result of this contact, the ejaculate can become contaminated with *Campylobacter* and numerous other bacteria providing a possible route of transfer to the eggs (Vizzier-Thaxton et al., 2005). While vertical transmission seems to be a viable method of transport for *Campylobacter*. Callicot et al. (2006), reported no significant amount of vertical transmission between parent and progeny flocks. M. Doyle (1984) reported that while *Campylobacter* could be recovered from the outside of eggs no *Campylobacter* was able to penetrate the intact eggshell. In contrast to these studies, Cox et al. (2002) showed that *Campylobacter* isolated from breeder hens and their progeny were clones of each other and that vertical transmission from hen to offspring is possible. It has been shown, that broiler

chicks less than 7 day old require much higher doses of Campylobacter to cause colonization compared to 21 day old broilers. The researchers attributed this to *Campylobacter* specific maternal antibodies present in 3 day old chicks (Sahin et al., 2003).

**Cross Contamination in Processing Plants**

A major concern for the poultry industry is cross contamination of *Campylobacter* positive birds to birds free of *Campylobacter*. One mode of transmission from bird to bird can occur during transportation from the farm to the processing plant. To transport birds from the farm to the processing plant, birds are placed in crates, and moved by truck to the processing plant. These crates are used multiple times, and even though they are cleaned between uses, these crates can become contaminated with *Campylobacter* by a positive flock and then when taken to another farm contaminate that group of birds also (Slader et al., 2002). A study by Berrang et al. (2004), indicated that *Campylobacter* from the feces in used transport crates could only be recovered by incubation in enrichment broth and then plating after storage. Transport stress has also been shown to increase the prevalence and number of *Campylobacter* on already colonized birds (Stern et al., 1995). Batch depletion, the removal of a portion of the birds from the house, while not common in the United States is another stressor that can increase *Campylobacter* numbers in poultry (Hald et al., 2001).

Feathers are commonly contaminated with bedding material and feces and thus *Campylobacter*. In the processing plant birds must pass through a hot

water bath or scalder to relax feather muscles for ease of feather removal. Scalder water can become contaminated with *Campylobacter* and contaminate successive carcasses. However the water temperature in the scalder can have a significant effect on the number of bacteria present, as higher temperatures result in lower counts (Salvik et al., 1994). Another machine, the feather picker that removes the feathers, has been shown to actually increase the number of *Campylobacter* bacteria on a carcass (Genigeorigs et al., 1986). Franchin et al. (2007) found over half of broiler carcasses were positive for *Campylobacter*, after evisceration, chilling and even 64 % after freezing, concluding that the only was to ensure safe poultry was by cooking.

### *Campylobacter* Colonization by Litter

The transmission of *Campylobacter* from litter or bedding is of particular interest as, in the U.S., it is common practice to reuse litter in a poultry house over multiple flocks (Chamblee et al., 2002). Because *Campylobacter* is excreted in fecal droppings and birds are in constant contact with fecal dropping deposited on litter, the potential for *Campylobacter* to be present in the litter from one flock to the next is high. While new litter is not normally contaminated with *Campylobacter* (Newell et al., 2003) it can be recovered from used litter. Montrose et al. (1984) investigated autoclaved and non-autoclaved litter, that was inoculated with *Campylobacter,* to reveal that the litter was capable of colonizing previously *Campylobacter* free broilers, thus providing evidence for litter as a viable pathway for flock colonization. In another experiment, it was

shown that birds on litter had almost twice the incidence of *Campylobacter* compared to caged birds (Willis et al., 2002). Both caged and floor reared birds were housed for a year and for the last four months of the study no *Campylobacter* was recovered from the birds in cages, while birds on litter continued to shed *Campylobacter* for the duration of the experiment (Willis et al., 2002). Kiess et al. (2007) found that turkeys grown on used litter had lower *Campylobacter* detection rates than turkeys grown on fresh litter, citing that the micro flora already present could compete with, and thus prevent the newly introduced *Campylobacter* from colonizing.

In other litter studies, pH levels were examined to see if different levels of pH could help reduce the presence of *Campylobacter*. Litter treatments, while used principally to lower ammonia levels in broiler houses, have been shown to also reduce the numbers of *E. coli*, *Salmonella* and *Campylobacter* while birds are present on the litter (Line, 2002; Pope et al., 2000). Line (2002) demonstrated that different levels of aluminum sulfate and sodium bisulfate significantly reduced *Campylobacter* numbers and high levels of aluminum sulfate yielded no recoverable *Campylobacter*. Poultry Litter Treatment®, made up of sodium bisulfite,' has been shown to reduce pH and significantly lower bacterial numbers especially *Campylobacter*, in used litter (Pope et al., 2000). Turnbull et al., (1973) investigated the effect of pH levels in used poultry litter and found that pH above 8.5 significantly reduced *Salmonella* numbers in litter, and that a pH of less that 6.5 had no effect on the pathogen. A study by Payne et al. (2007) showed that there are qualities of litter specific for pH and water activity,

which can affect the growth or death of bacteria. As the pH and water activity were changed to less desirable levels for bacterial growth, there was a significant decrease in the concentration of Salmonella (Payne et al., 2007).

**Litter Nutrients**

Another important part to supporting bacterial growth in litter is the availability of nutrients needed for survival. Poultry litter is rich in nutrients making it an excellent environment for bacteria. Bowers et al. (2001) looked at the nutrient buildup over 20 successive flocks in both pine shavings and sand. The study looked at available nitrogen, phosphorus, potassium, calcium, magnesium, ash, zinc, manganese, iron and copper. The results of this study indicated that over time the sand litter maintained less of these nutrients than pine shavings, concluding that sand litter could be used as a litter source for longer lengths of time as opposed to pine shavings. However, they concluded that the presence of fewer nutrients would be detrimental to sand when used as fertilizer (Bowers et al., 2001). Reduced nutrient content might also have a negative effect on the ability of bacteria to grow in sand litter.

**Effects of Moisture on *Campylobacter***

Amount of moisture can have a profound effect on Campylobacter, as well as many other species of bacteria. Buck and Kelly (1981), showed that on Columbia blood agar plates, there was a distinct difference in colony morphology between fresh plates and plates that had been incubated for 24 and 48 hours prior to inoculation. On fresh plates colonies were flat, grayish, spreading with an irregular shape incubated plates produces round convex butyrous or buttery type colonies (Buck et al., 1981). Relative humidity of a house, can also have impact on *Campylobacter* colonization of birds. Chicks placed on new litter contaminated with *Campylobacter* had delayed colonization in a lower relative humidity environment (30%) as compared to a high humidity environment (80%), this study also found a delayed colonization for delayed placement of chicks (Line 2006).

Water activity ($a_w$), the amount of water available for microbial use, is defined as the vapor pressure of the solution divided by the vapor pressure of the pure solvent. Bacteria need free or unbound water to sustain life, usually water activities of 0.90 or greater are needed for bacterial proliferation (Brown, 1976).

With such large quantities of used broiler litter produced annually, up to 1.73 dry tons/1000 birds (Patterson et al., 1998) utilization of this used litter is a major concern for poultry producers and growers. Broiler litter is used for a variety of purposes ranging from cattle feed (Cross et al., 1978; Jeffrey et al., 1998) to fertilizer (Nicholson et al., 2005) and most often reused in the same broiler house for sequential broiler flocks (Chamblee et al., 2002). With many

studies indicating that broiler litter can support large numbers of bacterial populations including pathogenic bacteria, someway of significantly reducing these bacteria is needed (Kelley et al., 1995; Kelley et al., 1998; Hutchinson et al., 2005; Montrose et al., 1984; Willis et al., 2002).

# CHAPTER THREE

# MATERIALS AND METHODS

## Survey of Arkansas Broiler Litter for *Campylobacter*

This experiment was a preliminary survey, of commercial broiler litter, to determine the prevalence of *Campylobacter* in litter and if any broiler house conditions might affect *Campylobacter* numbers. Litter samples of approximately 2 lbs, were collected from 27 commercial broiler houses located in northern Arkansas, the day of or, the day after catching. All farms were owned by same company, and 2 individuals were responsible for collecting all samples. This ensured the same house management and litter collection practices. Three sites within the house that were deemed to have the highest concentration of birds (drinker lines, feed lines, cool cells, fans or brooders) were sampled, and a composite was made from these three samples for analysis. All samples were collected during October of 2006. The litter samples consisted of pine shavings (3), rice hulls (18), sand (3) or a mix (3) of pine shavings and rice hulls (the amount of each in the mix was impossible to determine).

For each litter sample, 50 g was placed into 150 mL of *Campylobacter* Enrichment Broth. Samples were incubated at 42 C for 48 hours in a micro aerobic atmosphere. Micro aerobic atmosphere was achieved using an

Anoxamat[1] system which distributed an atmosphere of 6% $O_2$, 10% $CO_2$ and 84% $N_2$, into a Mart Anaerobic Canister. After a 48 hour incubation period, samples were diluted by a factor of 10, and 0.1 mL of broth was pippeted onto *Campylobacter* Cefex plates (5) and spread using a glass rod spreader. One hundred micro liters of the undiluted culture was also spread plated onto *Campylobacter* Cefex plates (5). These plates were incubated at 42 C for 48 hours in a micro aerobic atmosphere. After a 48 hour incubation, plates with bacterial colonies were counted using the Spiral Biotech Color Q automatic plate counter.

Presumptive *Campylobacter* colonies were tested using Pan Bio™ Latex Agglutination Test[2], as per manufacturer's instructions. If a colony tested positive on the plate it was assumed that all other colonies on the plate were *Campylobacter*.

Litter pH was tested by placing 20 g of each litter sample into 50 mL of distilled water and mixed for 10-15 seconds. pH was then tested using an Accumet Portable AP61 pH meter[3].

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1. Anoxamat Mark II Mart Microbiology BV
2. Latex: CAMPY (jcl) Panbio, Inc. 9075 Guilford Rd., Columbia, MD 21046
3. Accumet XL60 pH meter Fisher Scientific

Litter moisture content was reported as the percent moisture of the litter. Ten grams of each litter sample were put into tared aluminum drying dishes and placed in a drying oven at 100 C for 24 h. Afterwards, samples were removed from oven and weighed again. The difference in the before and after weights was calculated as the amount of moisture in the litter.The two individuals collecting litter samples were also instructed to record age of flock, age of litter (in number of flocks), house temperature, litter temperature and if the litter had been treated with Poultry Litter Treatment® (PLT®). This information would help later to determine any differences among these parameters that might effect *Campylobacter*.

**Persistence of *Campylobacter jejuni* in Three Types of Used Broiler Litter: Pine Shavings, Rice Hulls and Sand.**

Litter Preparation

Litter samples that had been previously tested, in the Arkansas study, for *Campylobacter* and found to be *Campylobacter* free were used in the current study. One gram of each litter sample was placed into 9 mL tryptic soy broth, vortexed and then 0.1 mL was plated onto *Campylobacter* Cefex plates. The plates were incubated as described in the previous section, and after 48 hours, plates were checked for growth. Litter was tested for *Campylobacter* a second time to make sure that litter samples had not become contaminated with *Campylobacter* during handling.

Stock Culture

*Campylobacter* from a lypodisk[4] *Campylobacter jejuni* stock culture, as resuscitated in 50 ml centrifuge tubes using 30 ml of *Campylobacter* enrichment broth. The cultures were incubated in a micro aerobic environment at 42 C for 48 hours. After 48 hours, incubated cultures were centrifuged at 2000 rpm for 5 minutes. The broth was drawn off leaving the *Campylobacter* cells. Next, 50 ml of peptone water was added to the centrifuge tubes. The enrichment broth was removed so that the nutrients in

4. MicroBiologics Kwik-Stik Microorganisms C. Jejuni ATCC 33291

the enrichment broth would not affect the *Campylobacter* after it was inoculated into litter. This solution was then gently vortexed to resuspend the bacteria, without rupturing the cell membranes.

To determine if Campylobacter could be recovered from litter after inoculation, 25 g of each litter type was weighed into 6 sterilized containers, 5 replications and one negative control. To each replicate, 10 ml of resuspended *Campylobacter* culture was added by distributing culture onto litter while litter was stirred around, to evenly distribute the *Campylobacter*. A 1 g sample of each replicate and control was removed and placed into 9 ml of tryptic soy broth. Then, 0.1 ml of each sample was plated onto *Campylobacter* cefex plates (3) and incubated for 48 hours at 42 C in a micro aerobic atmosphere. After 48 hours, plates were removed from incubator and counted, to determine initial bacterial concentration.

After initial 1 g samples were obtained, containers with litter samples were covered with aluminum foil and placed in an incubator at 35 C. At 24, 48, 72 and 96 hours another 1 g sample was taken from each replicate and plated to determine *Campylobacter* concentration at these times.

After it was determined that experimentally inoculated Campylobacter could be recovered from litter, a growth curve for this pathogen was needed. To determine *Campylobacter* numbers in a larger quantity of litter, 6 wooden boxes measuring 2 ½" x 2 ½" x 4 ¼" were filled with the *Campylobacter* negative litter, 5 replicates and one negative control. The three types of litter used were sand, rice hulls and pine shavings. To each replicate 50 m of *Campylobacter* culture

was added by distributing resuspended cultures over litter while the litter was being stirred around to evenly distribute the *Campylobacter*. These boxes were stored at 35° C. A 1 g sample of each replicate and control was removed and placed into 9 ml of tryptic soy broth. One hundred micro liters of each sample was spread plated onto *Campylobacter* cefex plates (3) and incubated at 42 C for 48 hours in a micro aerobic atmosphere. After 48 hours, the plates were counted as initial bacterial concentration. Samples were collected at 24, 48, 72 and 96 hours post inoculation to determine *Campylobacter* numbers over time.

All cultures and plates were incubated in micro aerobic atmosphere at 42 C for 48 hours in Mart Anaerobic Canisters. A micro aerobic atmosphere of 6% $O_2$, 10% $CO_2$ and 84% $N_2$ was obtained by Anoxamat system.

Moisture and pH of each replicate were taken every 24 hours with the Kelway Soil© Acidity and Moisture Tester Model HB-2 by placing the tester into the litter and allowing 3 minutes for the tester to calibrate and determine the pH and moisture. The Kelway meter was tested against the AOAC method of moisture determination, by drying 10 g of litter sample, in an attempt to validate the ease and effectiveness of the Kelway meter.

**The Effect of Different Environmental Conditions on the Survivability of *Campylobacter* in Litter: Atmosphere, Moisture, Time.**

Objective

This experiment investigated if *Campylobacter* in litter was capable of persisting in a recoverable form in its ideal growth conditions: high moisture, and micro aerobic atmosphere (6% $O_2$, 10% $CO_2$ and 84% $N_2$). If Campylobacter can not survive under these ideal conditions, then it is unlikely that the pathogen can survive in normal broiler house conditions.

Experimental Design

The following experiment was a randomized complete block with a split plot in time design. A 2 x 2 factorial arrangement of treatments over 5 time periods yielded a total of 20 treatments. The treatments for this experiment consisted of atmosphere (a): micro aerobic and aerobic; litter moisture (b): high and low; and time post inoculation with *Campylobacter* (c): 0 h; 6 h; 12h; 24 h; 120 h. Each treatment was replicated by repeating the entire trial to create 2 blocks. Treatment combinations were as follows: aerobic and low moisture; aerobic and high moisture; micro aerobic and low moisture; micro aerobic and high moisture.

Media

The media used in this experiment were *Campylobacter* Cefex agar, *Campylobacter* enrichment broth, peptone water and tryptic soy agar. *Campylobacter* Cefex agar and *Campylobacter* enrichment broth were prepared according to instruction from Dr. N. J. Stern (USDA Russell Research Center; Athens, GA). The peptone water and tryptic soy agar were prepared according to manufacturers' instructions.

Sample Preparation

Eight litter samples were prepared first, 4 samples for a micro aerobic environment and 4 samples for an aerobic environment. Each sample contained approximately 500 g of litter; 165 g was required for sampling. Therefore, the excess of litter should negate alteration of measured parameters due to sampling. Each litter sample was tested for pH, moisture content, water activity, *Campylobacter* concentration, total aerobes and total microaerophiles, at the appropriate time post inoculation.

The initial step in preparing the litter was removal of ammonia. To accomplish this litter was placed in a 35 C oven and 100 mL of water was added to the litter as necessary to avoid drying out and to aid in the release of ammonia. Ammonia measurements were taken once an hour using the Honeywell EC-P2[5].

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5. Honeywell EC-P2 Honeywell Analytics Inc.

Once ammonia levels reached 0 ppm, the amount of ammonia present in the litter was considered negligible. This was done so that there would not be an excess build up of ammonia in the Mart Anaerobic Canisters that might not occur in the open aerobic environment.

High moisture litter for aerobic and micro aerobic treatments was achieved by the addition of 200 mL of sterile water, to each sample. Low moisture samples received no additional water.

The litter samples that were in a micro aerobic atmosphere were stored in Mart anaerobic atmosphere canisters. The micro aerobic atmosphere (6% $O_2$) was achieved by using the Anoxamat system. For the litter samples that were in a total aerobic atmosphere treatment, the air inside each incubator was considered aerobic and nothing further was done to the litter. Each sample was kept in 4" S-40 PVC pipe that was cut 8" tall with a bottom only.

At the start of the experiment, all litter samples were inoculated with a $10^8$ cfu/ml dose of *Campylobacter jejuni*. *Campylobacter jejuni* was grown from a stock culture in *Campylobacter* enrichment broth. After incubation, cultures were centrifuged for 5 minutes at 2000 rpm. *Campylobacter* enrichment broth was drawn off and replaced with peptone water. Again, this procedure was to ensure that any nutrients present in the enrichment broth would not affect *Campylobacter* once it was inoculated onto the litter.

Measured Litter Parameter Procedures

*Campylobacter* concentrations were determined by placing 1 g of each litter sample into 9 ml of peptone water. One hundred micro liters of this dilution was spread plated onto *Campylobacter* Cefex plates (2). These plates were put in Mart Anaerobic Canisters, and then flushed with a micro aerobic atmosphere (6% $O_2$, 10% $CO_2$, and 84% $N_2$) via the Anoxamat system. The *Campylobacter* Cefex plates were put in a 42 C incubator and incubated for 48 hours. After 48 hours, plates were removed from the canisters and examined for potential *Campylobacter* colonies and then counted if possible. A potential positive colony from each plate was confirmed to be *Campylobacter* by the PanBio *Campylobacter* Latex Agglutination Test.

Total aerobic concentrations were determined by taking a 1 g sample from each litter replicate and serially diluting it to $10^{-6}$ in peptone water. Afterwards, 0.1 ml of diluent was plated onto tryptic soy agar plates (2) and incubated in aerobic conditions at 37 C for 24 hours. After incubation, plates were counted for total aerobes.

A 1 g sample from each litter replicate was serially diluted to $10^{-6}$ in peptone water to determine microaerophilic concentrations at 37 C. One hundred micro liters of this diluent was then spread plated onto tryptic soy agar plates (2) and placed into Mart Anaerobic atmosphere canisters. A micro aerobic atmosphere was achieved via the Anaxomat system (6% $O_2$, 10% $CO_2$, 84% $N_2$). Plates were incubated at 37 C for 24 hours and, after incubation, plates were removed from the canisters and counted.

Litter pH was measure with the pH Accumet XL60[5]. Ten grams of each litter replicate was placed in 100 ml of deionized water. These samples were mixed and allowed 5 minutes to equilibrate, after the pH measurement was collected.

Moisture content was determined by taking 10 g of litter from each litter replicate and placing it into a tared aluminum drying dish. The litter and dish were then placed in a 110 C drying oven for 24 hours. After 24 hours litter and dishes were removed and weighed. The difference in combined weight of dish and litter after removal from oven was considered to be the moisture content of the litter.

Water activity was analyzed using a HygroPalm AW1[6] water activity meter. Enough litter was taken from each sample to fill a 40 mm analysis cup and water activity was then analyzed via the water activity meter. This litter could not be reported in weight as higher moisture litter samples would take more litter to fill an analysis cup.

6. HygroPalm AW1 Portable Water Activity Meter rotronic instrument corp

**Instructions for Preparation of *Campylobacter* Cefex agar and *Campylobacter* enrichment broth via personal correspondence with Dr. N. J. Stern. (USDA Athens, GA)**

Campylobacter cefex agar (1 liter)

| | |
|---|---|
| Brucella Agar | 43 g |
| Ferrous Sulfate | 0.5 g |
| Sodium Bisulfite | 0.2 g |
| Pyruvic Acid | 0.5 ml |
| Distilled water | 950 ml |
| Cycloheximide | 1 ml |
| Cefeperazone | 1 ml |
| Lysed Horse Blood | 50 ml |

For preparation of 1 L of *Campylobacter* Cefex agar, 43 g of *Brucella* agar, 0.5 g ferrous sulfate, 0.2 g sodium bisulfite and 0.5 ml of pyruvic acid were added to 950 ml of distilled water, this was thoroughly mixed and boiled. The agar was autoclaved for 15 minutes at 121 C. After removal from autoclave, agar was cooled to 50 C. Once agar reached 50 C, 1 ml of cycloheximide, 1 ml of cefeperazone, and 50 ml of lysed horse blood were added and mixed thorougly. The agar was now ready to be equally dispensed into Petri dishes.

To prepare cefeperazone, 10 ml of sterile distilled water was added per 1 g of cefeperazone. After the solution dissolved, 1 ml was pippeted into cryovial tubes, and stored in a –80° C freezer.

To prepare cycloheximide, 5 ml of distilled water and 5 ml of methanol were added per 2 g of cycloheximide. The solution was filter sterilized and 1 ml was pippeted into storage tubes.

Campylobacter Enrichment broth (1 liter)

| | |
|---|---|
| *Campylobacter* Enrichment Broth | 27.6 g |
| Distilled Water | 950 ml |
| Lysed Horse Blood | 50 ml |
| Boltons *Campylobacter* Selective Supplement | 2 vials |

For preparation of 1 liter of *Campylobacter* enrichment broth, 27.6 g of *Campylobacter* enrichment broth was added to 950 ml of distilled water and autoclaved at 121 C for 15 minutes. After removal from the autoclave, the broth was cooled to 50 C, then 50 m of lysed horse blood, and 2 vials of Bolton's *Campylobacter* selective supplement were added.

For preparation of 1 vial of Bolton's Campylobacter selective supplement, 1 ml of acetone and 1 ml of sterile distilled water were added and mixed thoroughly with 1 g of supplement.

## Statistical Analysis

All data from previously described experiments were analyzed using SAS version 9.1 with one way ANOVA and the General Linear Models procedure at $\alpha<0.05$ level of significance. Statistics for the Kelway moisture meter validation were analyzed using SAS 9.1 Correlation procedure plotting Kelway versus AOAC moisture.

# CHAPTER FOUR

# RESULTS

## Survey of Arkansas Broiler Litter for *Campylobacter*

The results from the Arkansas litter survey indicated that 40% of all litter samples were positive for *Campylobacter* contamination. Of the positive samples, 36% were used litter, from at least 2 flocks. Sixty four percent of positive samples had been treated with PLT®. There were no significant differences in percent moisture, pH, house temperature and litter temperature between *Campylobacter* positive and negative litter. The average results of colony forming units after enrichment (cfu x $10^3$/g), pH, % moisture and numbers of samples from each litter type are listed in Table 1.

Only sand type litter samples had no recoverable *Campylobacter*. Sand litter had the lowest house temperature as well as the lowest moisture levels. Sand litter had the lowest litter temperature, but was not significantly lower than rice hulls or the mix type litter. Sand had significantly lower moisture than pine shavings and the mix type litter.

The average flock age at sample collection was 44 days, ranging between a minimum of 33 and a maximum 55 days. The age of used litter samples ranged between two flocks and up to one year, or approximately 5 flocks for sand samples.

Table 1 Comparison of cfu/g, Moisture, pH, Litter Temperature and House Temperature from Arkansas Survey[1,2,3]

| Litter Type | Cfu $x10^3$/g | % Moisture | pH | Litter Temperature (C) | House Temperature (C) |
|---|---|---|---|---|---|
| Rice hulls (18) | 6.7 | $28^{ab}$ | 8.66 | $26.2^{ab}$ | $22.7^{a}$ |
| Pine Shavings (3) | 15.7 | $29^{a}$ | 8.61 | $26.6^{a}$ | $22.7^{a}$ |
| Sand (3) | 0 | $25^{b}$ | 8.60 | $22.5^{b}$ | $18.6^{b}$ |
| Rice hulls & pine shavings (3) | 4.3 | $30^{a}$ | 8.57 | $24.7^{ab}$ | $22.9^{a}$ |
| SEM | $1.8x10^3$ | 1.8 | 0.2 | 1.3 | 1.6 |

1. Numbers with the same superscript are not significantlyT ifferent (α<0.1)T
2. cfu/g = colony forming units per gram of litter
3. cfu/g is concentration of Campylobacter after enrichment in Campylobacter enrichment broth for 48 hours and the plated onto Campy cefex agar and incubated for another 48 hours and counted. However these are not true concentrations of Campylobacter in litter but, no recoverable Campylobacter in sand litter is substantial. Litter and house temperatures are averages of numbers reported by people collecting litter.

**Persistence of Campylobacter jejuni in Three Types of Used Broiler Litter: Pine Shavings, Rice Hulls and Sand**

In the 25 grams of litter sample experiment, for all 3 types of litter and all replicates an average of $10^5$ colony forming units of *Campylobacter* were recovered at the initial sampling (Table 2). At the 24 hour sampling period no *Campylobacter* was recovered from any of the types of litter (Table 2). Therefore, at all other sampling times there was no significant difference among litter types with respect to cfu/g litter.

In the wooden box experiment, at the initial sampling time (0 hours) post inoculation, *Campylobacter* was recovered from all replicates and litter treatments at an average of $10^5$ cfu/g litter (Table 2). At the 24 hour sampling period, no *Campylobacter* was recovered from any of the replicates; this persisted for the remainder of the experiment for all three litter types (Table 2). There was no significant difference between the types of litter for all sampling times ($\alpha$<.05). For all treatments no *Campylobacter* was recovered from the negative controls.

Results from the Kelway moisture meter validation testing are shown in Figure 1. When Kelway was correlated to AOAC moisture an r value of 0.48 was found.

Table 2 *Campylobacter* Recovery Levels for All Types of Litter in cfu/g[1]

| Hours Post Inoculation | Rice Hulls | Sand | Pine Shavings |
|---|---|---|---|
| 0 | $10^{5,a}$ | $10^{5,a}$ | $10^{5,a}$ |
| 24 | $0^{b}$ | $0^{b}$ | $0^{b}$ |
| 48 | $0^{b}$ | $0^{b}$ | $0^{b}$ |
| 72 | $0^{b}$ | $0^{b}$ | $0^{b}$ |
| 96 | $0^{b}$ | $0^{b}$ | $0^{b}$ |

1. Numbers with the same subscript are not significantly different ($\alpha<0.05$)

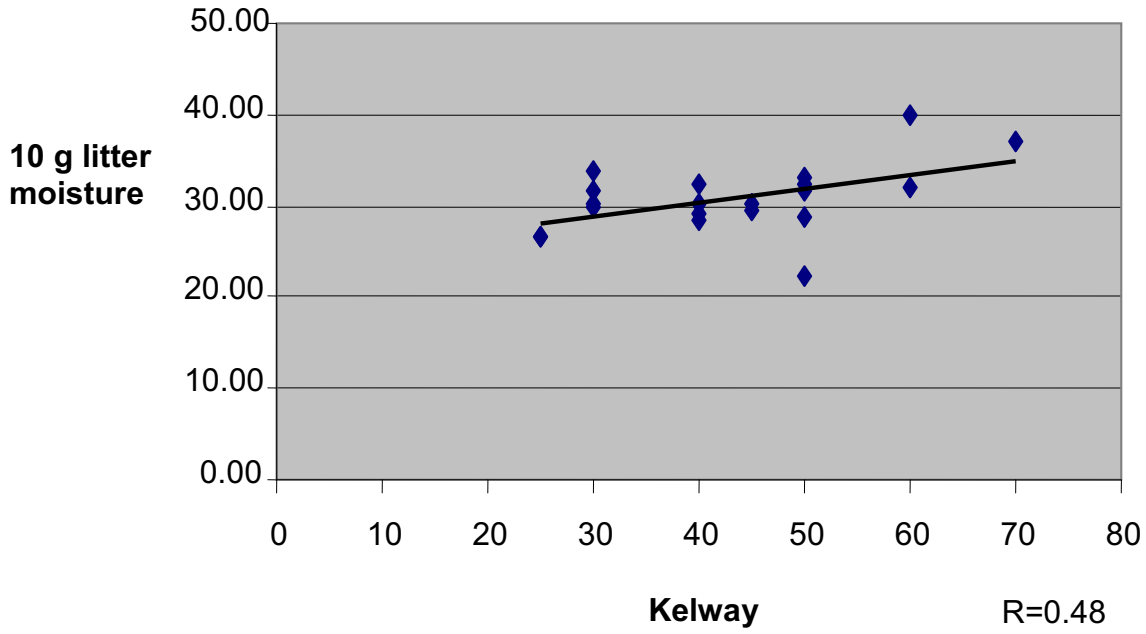


Figure 1 Comparison of Moisture found by Kelway to 10 grams litter dried at 100° C for 24 hours

**The Effect of Different Environmental Conditions on the Survivability of *Campylobacter* in Litter: Atmosphere, Moisture, Time.**

In both blocks of this experiment, *Campylobacter* was recovered from all treatments and samples at the initial (0 hour) sampling time (Table 3). At the 6 hour, and all subsequent sampling, no *Campylobacter* was recovered from the aerobic low moisture treatment. In the first block, *Campylobacter* was not recovered from the micro aerobic low moisture treatment at the 6 hour time period, but was recoverable in the second block, at the 6 hour time. In both trials, *Campylobacter* was recovered from the high moisture treatments for both atmospheres at 6 hours of litter incubation.

At the 12 hour sampling period, all plates for the micro aerobic, high moisture treatment were positive for *Campylobacter* growth while no other treatments yielded recoverable *Campylobacter*. At the 24 hour sampling only one plate from the micro aerobic, high moisture treatment was positive for *Campylobacter* in the second block, while no *Campylobacter* positive plates were present in the first block. At the 120 hour sampling times no treatments were positive for *Campylobacter* in either trial.

There was a significant three way interaction for positive litter samples due to, atmosphere, moisture and time ($p<0.0052$). At the 6 hour sampling time, the number of litter samples positive for Campylobacter with high moisture, regardless of atmosphere, were significantly higher than the low moisture treatments with the aerobic, low moisture treatment yielding no growth. At the 12

hour sampling time, micro aerobic high moisture treatment had significantly higher *Campylobacter* positive litter samples than all other treatments. At the 24 hour sampling time, even though one sample was positive for *Campylobacter* for the micro aerobic high moisture treatment there was no significant difference between this treatment, and all other treatments for the recovery of *Campylobacter*.

For pH and measured moisture, there was a significant main effect due to atmospheric conditions. Aerobic treatments yielded significantly higher pH than micro aerobic atmosphere treatments, 8.89 and 8.37 respectively (Table 4). Also, overall litter moisture for micro aerobic treatments was significantly higher than aerobic treatments with mean litter moistures of 58 % and 54 % respectively (Table 4).

For ph and measured moisture, there was a significant main effect due to moisture treatment (Table 4). The no added moisture treatment had a significantly higher mean pH value while the high moisture treatment had an overall higher moisture level.

The main effect of time yielded a significant effect on overall pH with the 0 or initial sampling period having the highest pH and then it decreased reaching the lowest pH at 120 hours (Table 5). Time also had a significant effect on moisture, with the 0 hour sampling having the highest overall mean moisture, and the 120 hour sampling time having significantly lower moisture (Table 5). Time also affected $a_w$, at the 0, 6, 12 and 24 hours sampling period saturated water

activites were not different, but at 120 hours the $a_w$ was found to be significantly lower (Table 5).

For pH, there was a significant three way interaction between atmosphere, moisture and time as shown in Figure 2 and Table 6 ($p<0.0001$, sem +/- 0.03, lsd = 0.0876, n=2). At the initial or 0 hour sampling there was no significant difference in pH for all treatments. Starting at the 6 hour sampling, micro aerobic atmospheres began to have significantly lower pH value as compared to aerobic treatments. Additionally, the micro aerobic, high moisture treatment yielded lower pH values than the micro aerobic, low moisture treatment. Finally, at 120 hours pH for the aerobic, low moisture treatment was less than that of the aerobic, high moisture treatment.

For litter moisture there was a significant 3 way interaction between atmosphere, moisture and time, shown in Figure 3 and Table 6 ($p<.0625$, sem +/- 3.4, lsd= 5.485, n=2). Litter moisture was consistently higher for the two high moisture treatments as compared to the two low moisture treatments. Also, there was no significant difference in moisture between the two high level moisture treatments and between the two low level treatments until the 120 hour sampling time when all treatments had significantly different moisture levels ($\alpha<.05$). The lowest moisture level was for the aerobic and low moisture level treatment at the 120 hours sampling time.

Table 3 Number of *Campylobacter* Cefex Plates Positive for *Campylobacter* Growth[1,2,3,4]

| | Time (hours) | | | | |
|---|---|---|---|---|---|
| Atmosphere, Moisture | 0 | 6 | 12 | 24 | 120 |
| Aerobic, Low | 8/8[a] | 0/8[c] | 0/8[c] | 0/8[c] | 0/8[c] |
| Aerobic, High | 8/8[a] | 8/8[a] | 0/8[c] | 0/8[c] | 0/8[c] |
| Micro aerobic, low | 8/8[a] | 4/8[b] | 0/8[c] | 0/8[c] | 0/8[c] |
| Micro aerobic, high | 8/8[a] | 8/8[a] | 8/8[a] | 1/8[c] | 0/8[c] |

1. Single Colony from each plate was confirmed through Pan Bio *Campylobacter* Latex Agglutination Test
2. Most Plates were too numerous too count or had confluent growth less than half of the plates positive for *Campylobacter* growth were countable. So, plates were just described as either positive or negative for *Campylobacter* growth. Potential *Campylobacter* colonies were tested for *Campylobacter* with Latex Agglutination Test.
3. Numbers of positive plates with the same superscript are not significantly different ($\alpha<0.05$). Significant three way interaction between atmosphere, moisture and time ($p<0.0052$) for *Campylobacter* positive samples.
4. sem +/- 11%

Table 4 Significant Main Effects Due to Atmosphere and Moisture for Litter pH and Litter Moisture[1]

| | Atmosphere | | Moisture Level | |
|---|---|---|---|---|
| | Aerobic | Micro aerobic | No Added | 200 mL Added |
| pH[2] | 8.83[a] | 8.37[b] | 8.64[a] | 8.57[b] |
| Moisture[3] | 54.59[b] | 58.84[a] | 50.05[b] | 63.39[a] |

1. Numbers with the same superscript are not significantly different for pH and moisture ($\alpha<0.05$).T
2. SEM +/-.008 lsd=0.0369 n=20
3. SEM +/-.9 lsd=4.1958 n=20

Table 5 Overall Litter pH, Moisture and $a_w$ Significant Differences due to Time[1,2,3,4]

| Time (Hours) | pH[3] | Moisture[4] | $A_w$ |
|---|---|---|---|
| 0 | 8.90[a] | 62.80[a] | 1.00181[a] |
| 6 | 8.73[b] | 58.60[b] | 1.00000[a] |
| 12 | 8.67[c] | 57.65[b,c] | 1.00000[a] |
| 24 | 8.59[d] | 55.81[c] | 1.00000[a] |
| 120 | 8.13[e] | 48.72[d] | 0.97244[b] |
| SEM | 0.01 | 0.9 | 0.008 |

1. Numbers with the same superscript are not significantly different (α<.05)
2. The gradual decline of all measured litter qualities are shown, regardless of treatment. pH had the most noticeable decline with levels at all sampling times being significantly less than the previous sampling time.
3. p<0.0001
4. p<0.0001

Table 6 Significant Three Way Interaction for Litter pH and Moisture due to Treatments: Atmosphere, Moisture and Time[1,2,3,4]

| Litter Treatment | | | | | |
|---|---|---|---|---|---|
| Atmosphere | Moisture | Time | pH[2] | Moisture[3] | $a_w$[4] |
| Aerobic | Low | 0 | 8.93[a] | 58.58[b] | 1.00 |
| Aerobic | High | 0 | 8.89[a] | 66.78[a] | 1.00 |
| Micro aerobic | Low | 0 | 8.91[a] | 58.43[b] | 1.00 |
| Micro aerobic | High | 0 | 8.88[a] | 67.43[a] | 1.00 |
| Aerobic | Low | 6 | 8.83[a] | 52.72[b] | 1.00 |
| Aerobic | High | 6 | 8.73[b] | 64.86[a] | 1.00 |
| Micro aerobic | Low | 6 | 8.77[ab] | 51.53[b] | 1.00 |
| Micro aerobic | High | 6 | 8.58[c] | 65.28[a] | 1.00 |
| Aerobic | Low | 12 | 8.85[a] | 49.93[b] | 1.00 |
| Aerobic | High | 12 | 8.83[a] | 63.94[a] | 1.00 |
| Micro aerobic | Low | 12 | 8.65[b] | 52.56[b] | 1.00 |
| Micro aerobic | High | 12 | 8.36[c] | 64.18[a] | 1.00 |
| Aerobic | Low | 24 | 8.94[a] | 46.95[b] | 1.00 |
| Aerobic | High | 24 | 8.95[a] | 60.73[a] | 1.00 |
| Micro aerobic | Low | 24 | 8.33[b] | 52.08[b] | 1.00 |
| Micro aerobic | High | 24 | 8.13[c] | 63.48[a] | 1.00 |
| Aerobic | Low | 120 | 8.51[b] | 27.40[d] | 0.90 |
| Aerobic | High | 120 | 8.91[a] | 54.04[b] | 0.99 |
| Micro aerobic | Low | 120 | 7.69[c] | 50.30[c] | 0.99 |
| Micro aerobic | High | 120 | 7.43[d] | 63.16[a] | 0.99 |
| SEM | | | 0.03 | 3.4 | 0.015 |

1. Numbers with the same superscript are not significantly different at that sampling time ($\alpha<0.05$)T
2. $p<.0001$; n=2
3. $p<0.0625$; n=2
4. There was no interaction or main effects with water activity, but numbers are reported anyway.

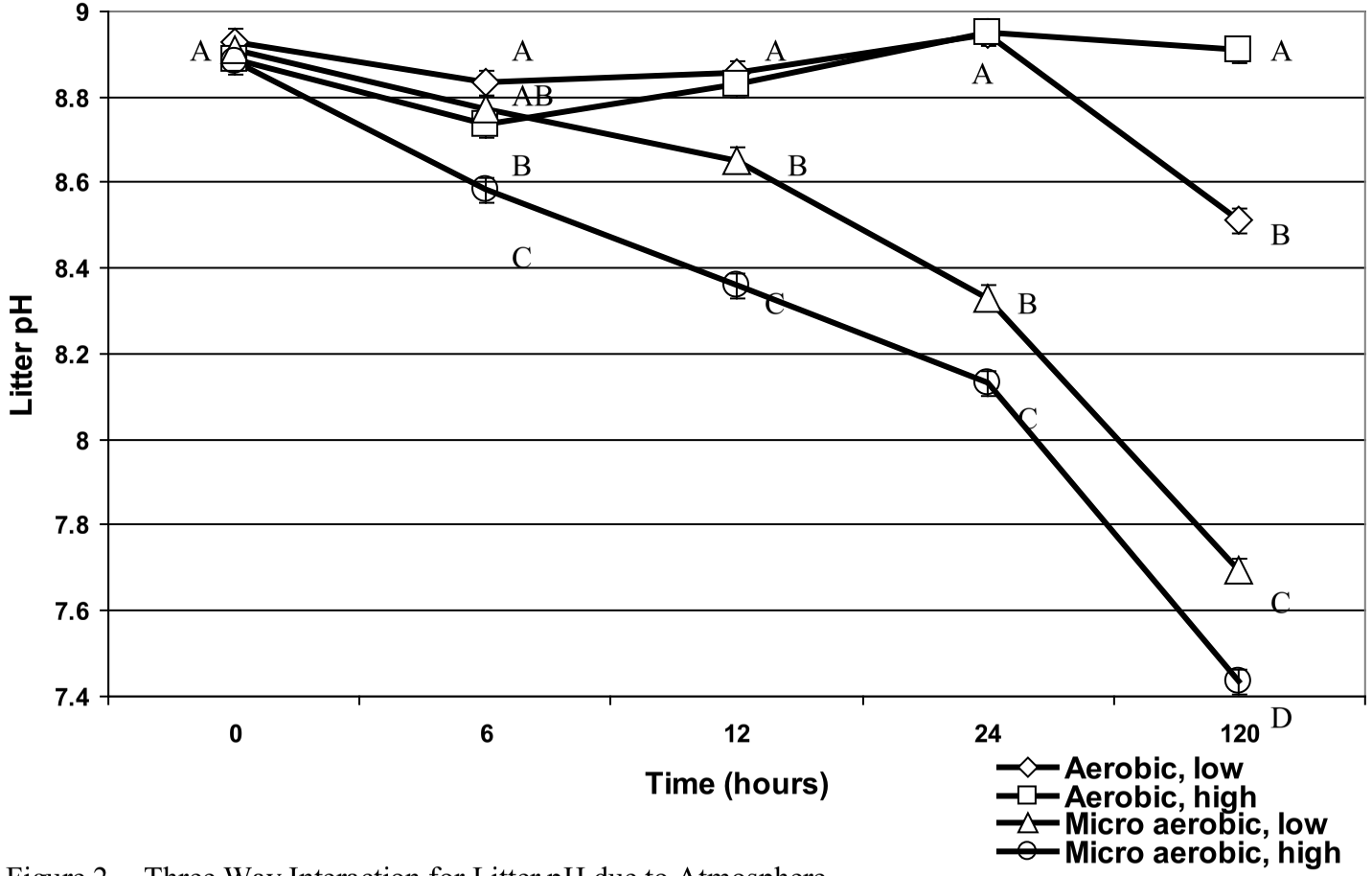


Figure 2 Three Way Interaction for Litter pH due to Atmosphere, Moisture and Time[1,2]

1. $p<0.0625$, SEM 3.4, LSD 5.5485
2. Numbers with the same superscript are not significantly different.

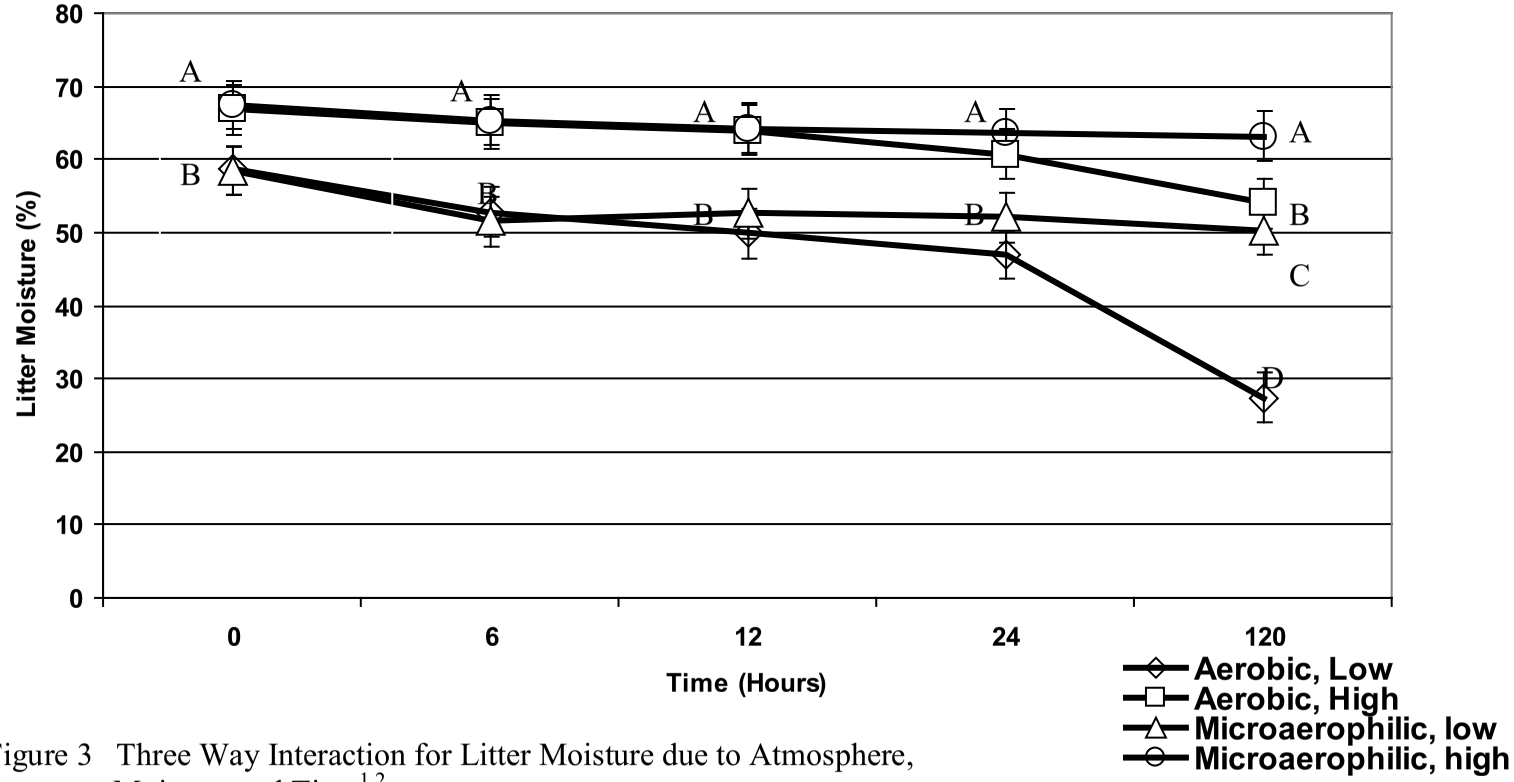


Figure 3 Three Way Interaction for Litter Moisture due to Atmosphere, Moisture and Time[1,2]

1. P<0.0625; SEM +/-3.4; lsd= 5.5485
2. Numbers with the same superscript are not significanty different ($\alpha<0.05$)

# CHAPTER FIVE

# DISCUSSION

In the survey of Arkansas broiler litter, no *Campylobacter* was recovered from any of the sand litter samples. In all cases the sand had been used longer than any of the other types of litter, by at least one year. The only significant differences in sand litter and the others were lower moisture content and lower house temperature. Pine shaving type litter had a significantly higher concentration of *Campylobacter* than either rice hulls or a mix of pine shaving and rice hull. Pine shavings also had a significantly higher amount of moisture than any of the other three types. These findings are in agreement with Asaniyan et al. (2007) and Macklin et al. (2005) who found used sand litter had significantly lower bacterial loads than pine shaving litter.

The difference in *Campylobacter* recovery between the types of litter is most likely due to moisture in the litter. Sand with its significantly lower amount of moisture had no recoverable *Campylobacter*, and pine shavings with the highest amount of moisture had significantly higher concentrations of *Campylobacter*. Macklin et al., (2005) also determined moisture levels in pine shaving litter to be significantly higher than sand type litter, and found that bacterial levels correspond to litter moisture levels.

Houses with pine shavings also had significantly higher house temperatures and litter temperatures. However, differences in house and litter temperatures were unlikely to be caused by differences in broiler house management practices as all samples were in the same division from one company. Sand releases water more rapidly than other types of litter and therefore cools faster possibly leading to a cooler house temperature and cooler litter temperature. If this is the case, then bacteria will not be able to acquire the necessary water for survival. The water in the sand crystal may also be bound water and therefore unavailable for use by bacteria. Also the nutrient content of sand litter is less than that of pine shaving litter (Bowers et al., 2003). With little moisture, little nutrients and cooler temperatures, the sand litter could be a harsh environment for *Campylobacter* and any other bacteria. The next experiments attempted to formulate a growth curve of Campylobacter in these three types of litter.

In experiments 2 and 3, 25 g sample and wooden boxes, the inability to recover Campylobacter at the 24 hour sampling time indicates that in conditions near that of broiler grow out houses the pathogen cannot survive. Even in a small sample quantity such as 25 g it would be difficult to not recover any *Campylobacter* that was present at the 24, 48, 72, or 96 hours post inoculation. As the 10 mL of Campylobacter culture added should ensure that all 25 g of litter was inoculated. These experiments also demonstrated that there is no difference in the three types of litter ability to grow *Campylobacter jejuni*. This is in contrast

to the Arkansas findings, but the Campylobacter recovered from these samples could be better adapted to environmental conditions in broiler houses.

In the experiment that investigated atmosphere, moisture and time as treatments, Campylobacter had a very short recoverable window of 12 hours that provided abundant growth. At 24 hours minimal growth occurred and it was at a concentration of only 300 cfu/g of litter on one sampling plate of the micro aerobic, high moisture treatment. The other micro aerobic treatment had significantly lower moisture content by 13.75 % at 6 hours, for this treatment *Campylobacter* was only recovered at 6 hours in block 2 and 0 hours in block 1. Also at 6 hours the pH for these two treatments was significantly different with the low moisture treatment having a higher ph than the high moisture treatment. This trend continued for the rest of the experiment. *Campylobacter* was recovered from the aerobic high moisture treatment up to 6 hours demonstrating that in a wet environment *Campylobacter* can persist for at least 6 hours even without optimal atmosphere, but was not recoverable at the 12 hour time. The moisture content of this litter was not significantly different from the micro aerobic high moisture treatment until the 120 hour sampling time. Indicating that, higher moisture can sustain Campylobacter, but that a micro aerobic atmosphere is also needed for longer viability (Holt et al., 1986).

The aerobic atmospheric treatments only had recoverable *Campylobacter* at the 6 hour time for high moisture treatment for both trials and were only recoverable in the low moisture treatment at the initial or 0 hour sampling time. For these treatments pH was significantly different at 6 and 120 hour sampling

times and was not significantly different at all other sampling times. Water activity for these treatments was not significantly different until 120 hours when low moisture was significantly less than high moisture.

This experiment demonstrated that in *Campylobacter*'s preferred growth environment (wetter litter in a micro aerobic environment), it can persist in litter for up to 24 hours in and up to 6 hours in drier litter under micro aerobic atmospheric conditions. Macklin et al (2008) also found that litter artificially inoculated with *Campylobacter* and then composted for 7 days either near the surface or inside the compost piles could not be recovered after 7 days.

Experiment 4, also demonstrates that in wetter litter conditions *Campylobacter* persists much better than in significantly drier litter despite differences in atmosphere. The ability of *Campylobacter* to survive in high moisture litter after 6 hours of incubation under aerobic conditions compared to Campylobacter survival in dry moisture litter incubated in a micro aerobic environment clearly demonstrates this. The lesser amount of moisture proved to have a significant effect on the survival of *Campylobacter*. J. E. Line (2006) also found significantly less chick colonization with Campylobacter, when housed in low relative humidity conditions. The litter collected for this experiment was used litter which had total number of aerobic and micro aerobic bacteria of 8.17 and 7.46 logs respectively. These bacteria could have out competed *Campylobacter* in the litter which would be in agreement with Kiess et al., 2007, who found lesser concentrations of *Campylobacter* in turkeys that were housed on used litter.

Figures 2, and 3 show the significant differences in pH, and moisture plotted as a function of time. As time increased there were significant differences in pH and moisture starting at the 6 hour sampling time, and these differences increased until the end of the experiment. An interesting note is the significant decrease in pH for the 2 micro aerobic treatments at 12 hours, 24 hours, and 120 hours. This decrease in pH could be caused by a build up of excess $CO_2$, thereby reducing the pH for the micro aerobic treatments which received 10 % $CO_2$ by the Anoxamat system. The significantly lower pH for micro aerobic high moisture treatment at 6 hours and beyond indicates that litter pH might have an effect on the growth of Campylobacter. The pH of the ceca ranges between 5.7 and 6.9 (Duke, 1986), with *Campylobacter* being well adapted to chicken intestines at this pH. The lower pH of the micro aerobic high moisture treatment could help sustain *Campylobacter* for longer periods of time. Turnbull et al. (1973) found that higher pH levels, around 8.5, significantly reduced *Salmonella* numbers in litter, and that a pH below 6.5 had no effect on *Salmonella*. Even though the pH levels in experiment 4 did not fall below 7, again the lower pH of the micro aerobic high moisture treatment could have sustained the *Campylobacter*, for a longer time. Line et al. (2002) found that when litter pH dropped below 4 there was no recoverable *Campylobacter* present. The significantly higher pH for aerobic atmosphere treatments and micro aerobic low moisture could have a harmful effect on *Campylobacter* deposited in litter.

No *Campylobacter* being recovered at 120 hours for all treatments shows that, with adequate down time, *Campylobacter* persistence can be halted in used

poultry litter. If the litter is allowed to dry out before reutilization it should cause enough harm to the bacteria to eradicate *Campylobacter* in litter and virtually eliminate the chance of carryover between successive flocks, litter used for fertilizer on crops, and litter used as cattle feed. In 2008, Macklin et al, artificially inoculated litter compost piles with *Campylobacter* and could not recover it after 7 days of composting which was also found in this experiment but without composting.

Further research is needed however to explain why *Campylobacter* can be recovered from certain litter samples from commercial grow out houses. Also research is needed to find minimum litter storage methods to reduce *Campylobacter* and other pathogens from litter before reutilization.

The goal of this research was to show if *Campylobacter* could persist in used broiler litter long enough to colonize the subsequent broiler flock. This research indicated that under ideal atmospheric conditions no culturable *Campylobacter* could be recovered after 24 hours of incubation and with over 60 % litter moisture. Even though, the amount of litter moisture in this study was much higher than that found in commercial broiler litter. It is doubtful that the moisture negatively affected the bacteria, because Cools et al. (2003) were able to recover *Campylobacter jejuni* inoculated into drinking water after 64 days of incubation. This conclusion is supported by the work that investigated different types of litter inoculated with *Campylobacter* and found no recoverable *Campylobacter* after 24 hours of incubation. These four experiments demonstrate that, given enough down time in broiler houses, *Campylobacter*

should not persist long enough to colonize a new flock of birds, without fresh live viable *Campylobacter* cells.

Past research has shown that after inoculated onto litter *Campylobacter* is capable of colonizing birds (Montrose et al., 1984). However, other research has shown that when birds are removed from litter and housed in cages the birds will eventually stop shedding *Campylobacter* (Willis et al., 2002).

This present thesis research suggests that without birds present in the house to shed fresh *Campylobacter* cells onto the litter, the litter itself is incapable of harboring the bacteria long enough to colonize sequential flocks. In agreement with Macklin et al. (2008) that 7 days of storage should be adequate to significantly reduce *Campylobacter* numbers. Even though, *Campylobacter* was recovered from commercial broiler litter in the Arkansas survey, those litter samples were collected at 24 hours or less. If longer time had lapsed between broiler catching and sample collection then the possibility of recovering *Campylobacter* would have been lower.

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