

Effects of Management on the Bacterial Community Present in Poultry Litter

by

Zachary Thomas Williams

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 4, 2012

Keywords: Poultry, litter, management, bacteria

Approved by

Kenneth S. Macklin, Chair, Associate Professor, Department of Poultry Science
John P. Blake, Professor, Department of Poultry Science
Joseph B. Hess, Professor, Department of Poultry Science
Mark R. Liles, Associate Professor, Department of Biological Sciences

Abstract

An unwanted byproduct of commercial broiler chicken grow out is thousands of tons of litter. This litter is the material on which the broiler chicken spends its entire life. Litter consists of a starting material, in the southeast United States normally pine shavings, plus excreta, feathers, feed, litter beetles, parasites and bacteria. While some litter is used for other applications, such as plant fertilizer or livestock feed, most of it is reused from one flock to the next. With proper management, the litter can be maintained in good condition for several years. A problem associated with litter is the high number of bacteria that survive in the litter environment. Most of these bacteria are not harmful, but some such as *Salmonella enterica*, can cause serious illness in humans through contaminated meat. Other bacteria, such as *Clostridium perfringens*, can cause illness in both chickens and humans. In both instances, the economical impact of such illness is substantial, reaching into the millions of dollars in losses.

The majority of previous research into litter bacterial makeup has utilized the hand grab method of sampling that simply removes the top inch or two of litter. From this sampling, researchers have extrapolated the bacterial makeup of the entire litter bed. However, most commercial broiler houses have more than two inches of litter. It is the author's belief that previous findings concerning the amount of bacteria present in litter could be erroneous. Therefore the first phase of this dissertation is to identify the concentrations of several pathogenic bacteria, their stratification from top to bottom of

the litter and location of the bacteria within the house. Sampling would include the entire depth of the litter bed, including the hard dirt pad underneath. This would be achieved utilizing traditional culture and second generation high throughput sequencing methods.

The second phase aims to discover any litter management techniques that would reduce the pathogenic bacteria load in litter, specifically *Salmonella*. In recent years, the emphasis on controlling this pathogen has switched from processing plant to more on farm control methods. The methodology to be examined will be the application of several commercial and novel litter amendments that would normally be used for ammonia reduction on a commercial poultry farm. These amendments consist of several acid salts, a liquid acid and several nonpathogenic bacteria. The acidic amendments function by lowering the pH of litter from its normal basic pH, around 8 to 9, to an acidic pH of 5 or less. This reduction in litter pH not only traps ammonia, but could reduce the concentration of pathogenic bacteria present in litter.

Acknowledgments

I would like to thank broilers everywhere.

Table of Contents

Abstract	ii
Acknowledgments	iv
List of Tables	viii
List of Figures	x
Introduction.....	1
Literature Review	4
Litter Production and Characteristics	4
Litter Microbiology.....	5
Distribution of Bacteria in Poultry Litter.....	10
Litter Management and Effects on Bacteria	12
Polymerase Chain Reaction	16
Pyrosequencing.....	17
Molecular Analysis of Poultry Litter	19
References.....	20
Stratification of bacterial concentrations, from upper to lower, in broiler litter	26
Abstract.....	26
Introduction.....	27
Materials and Methods.....	29
Results.....	32

Discussion	35
References	37
The Effect of Sodium Bisulfate on Salmonella Viability in Broiler Litter	49
Abstract	49
Introduction	50
Materials and Methods	52
Results	56
Discussion	60
References	63
Reduction of Salmonella and Ammonia Emissions in Broiler Litter Using Sulfuric Acid and Aluminum sulfate	71
Abstract	71
Introduction	72
Materials and Methods	74
Results	78
Discussion	81
References	83
Reducing ammonia volatilization and <i>Salmonella enterica</i> concentrations in poultry litter using <i>Bacillus subtilis</i>	93
Abstract	93
Introduction	94
Materials and Methods	96
Results	100

Discussion.....	102
References.....	104
Microbiological analysis of poultry litter using high throughput 16S rDNA sequencing.....	109
Abstract	109
Introduction	110
Materials and Methods.....	113
Results	116
Discussion.....	117
References.....	119

List of Tables

Table 1 Composite litter fraction results for preliminary Auburn University Poultry Farm	41
Table 2 Bacteria concentrations during 49 day broiler grow out.....	42
Table 3 Differences in broiler litter bacteria concentration at three difference locations within a commercial broiler grow out house; exhaust fans, middle of house, evaporative cooling cells	48
Table 4 Description of treatments used in experiments 3 and 4.....	65
Table 5 Average ammonia levels (ppm) for each treatment at 0, 24, 48, 72 and 96 hours for experiments 1 and 2.....	68
Table 6 Difference in <i>Salmonella</i> concentrations and litter pH for experiments 3 and 4.....	69
Table 7 Average ammonia levels (ppm) for each treatment at 0, 24, 48 72 and 96 hours after treatment with sulfuric acid	85
Table 8 Total aerobic, anaerobic and <i>Salmonella</i> concentrations at 0, 24 and 96 hours after treatment with sulfuric acid (\log_{10} cfu/g litter)	86
Table 9 Litter pH levels at 0, 24, and 96 hours after sulfuric acid application.....	87
Table 10 Litter moisture (percent) for litter treated with sulfuric acid.....	88
Table 11 Ammonia measurements (ppm) for litter treated with 22.7, 45.4 68 or 0 kg/92.9 m ² of aluminum sulfat.....	89
Table 12 Total aerobic, anaerobic and <i>Salmonella</i> concentrations (\log_{10} cfu/g litter) at 0, 24 and 96 hours after treatment aluminum sulfat	90
Table 13 Litter pH levels at 0, 24 and 96 hours after aluminum sulfate application.....	91
Table 14 Litter moisture (percent) for litter treated with aluminum sulfate.....	92
Table 15 Ammonia reduction in percent from time 0 for Experiment 1	105
Table 16 Bacteria enumeration results from Experiment 1 (\log_{10} cfu/g litter).....	106

Table 17 Ammonia measurements in percent reduction from time 0 for Experiment 2	107
Table 18 Comparison results of <i>Salmonella</i> recovery from Experiment 2 (log ₁₀ cfu/g litter).....	108
Table 19 Relative abundance of each family of bacteria in poultry litter.....	121

List of Figures

Figure 1 Graphical representation of a generic commercial broiler house	40
Figure 2 Decrease between top, middle and bottom fractions of litter for total aerobic bacteria	43
Figure 3 Differences between top, middle and bottom fraction of litter for total anaerobic bacteria	44
Figure 4 Differences between top, middle and bottom fraction of litter for <i>Staphylococcus</i> spp.	45
Figure 5 Differences between top, middle and bottom litter fractions for <i>E. coli</i>	46
Figure 6 Comparison of <i>C. perfringens</i> concentration in top, middle and bottom litter fractions	47
Figure 7 Salmonella concentrations and litter pH for experiments 1 and 2	66

Literature Review

Litter Production and Characteristics

Litter is comprised mainly of organic materials. Litter starts as bedding material, whether it's pine shavings, peanut hulls, rice hulls, sand or any other readily available material, with pine shavings being the most common in the southeastern United States. Bedding becomes litter after the chickens are placed onto it. The addition of chickens onto the litter adds large amounts of excreta, and varying amounts of feathers and feed.

Broiler litter contains sufficient levels of nitrogen, phosphorus and potassium to make it suitable as a fertilizer. In a survey of the quantities of these compounds in Alabama poultry litter, the average nitrogen content was found to be 4.0%, phosphorus 1.5% and potassium 2.3% with 24.9% crude protein. The researchers calculated the price of a ton of poultry litter, used as fertilizer, based on this composition at \$31.23 per ton, although this price has significantly increased since this article was published (Stephenson *et al.*, 1990). These numbers are in agreement with Martin and McCann (1998) who found phosphorus and potassium at 2.1 and 3.0% in Georgia poultry litter, with 27.9% crude protein. These researchers also found an average moisture content of 21.9%. Poultry litter pH trends towards the basic side, with more basic pH being beneficial to ammonia volatilization. In a survey of litter from 12 states in the United States, litter pH ranged from 6.6 to 9.0 with an average of 8.0 (Terzich *et al.*, 2000). All of these characteristics help bacteria persist and multiply in broiler litter.

Litter Microbiology

Poultry litter has been shown to harbor copious numbers of bacteria. Total litter bacteria concentrations fall within the range of 10^{10} to 10^{11} colony forming units (cfu) per gram of litter (Scheffererle 1965; Terzich *et al.*, 2000). Total aerobic bacteria counts are lower at 10^8 to 10^{10} (Macklin *et al.*, 2005; Lu *et al.*, 2003). These numbers can vary with the age of the litter and age of the birds, as shown by Macklin *et al.*, 2005. Fresh litter and bedding were found to have 10^5 cfu per gram, as soon as birds were placed on the litter the numbers of bacteria increased by several orders of magnitude to 10^8 . The bacterial numbers peaked at 10^{10} during week 6 for new bedding and week 4 for litter used for more than one flock. The researchers found that after aerobic bacteria peaked at 10^{10} cfu/gram of litter bacteria numbers either remained steady or declined until the end of each poultry flock grow-out. Levels of bacteria do not vary in the different regions of the United States. In twelve of the highest poultry production states, total bacterial numbers were found to lie between 10^9 and 10^{11} cfu/g of litter with the average being 10^{11} (Terzich *et al.*, 2000).

Bacteria found in broiler litter of most interest or concern to the poultry industry and the consumer include: *E. coli*, *Staphylococcus* spp., *Clostridium perfringens*, *Campylobacter* spp. and *Salmonella* spp. (Lu *et al.*, 2003; Alexander *et al.*, 1968; Bang *et al.*, 2002; Dhanarani *et al.*, 2009; Graham *et al.*, 2009; Terzich *et al.*, 2000; Vizzier-Thaxton *et al.*, 2003).

***Staphylococcus* spp.**

Staphylococcus is a genera of gram positive, nonmotile, facultative anaerobes, capable of producing enterotoxins in human and animal hosts (Bergey's Ninth Edition,

1994). *Staphylococcus* spp. have been routinely observed to be the highest percentage of the total bacterial population in litter. In 1968, Alexander *et al.*, found *Staphylococcus* in 44 out of 44 litter samples. Lu *et al.* (2003) found *Staphylococcus* accounted for 13% of total aerobes by culture methods and 8% by 16S rRNA sequencing. In comparison, Dhanarani *et al.* (2009) found that *Staphylococcus* made up 29.1% of the total bacteria present. *Staphylococcus* concentration numbers have been shown as high as 10^{11} cfu/g of litter (Omeira *et al.*, 2006; Terzich *et al.*, 2000). Antibiotic resistance in *Staphylococcus* makes it an important bacterium for investigation. Both Khan *et al.* (2002) and Graham *et al.* (2009) found antibiotic resistance genes in *Staphylococcus* isolated from poultry litter. Khan *et al.* (2002) found all isolates to be resistant to multiple antibiotics; however they did not find any *mrsA* (Methicilin resistant) genes.

S. aureus intoxication accounts for approximately 240,000 cases of human food-borne illness per year (Scallan *et al.*, 2011). *Staphylococcus* spp. are ubiquitous in the environment in and around poultry houses, and in poultry can cause serious infection in bones, joints, feet and organs if they become septic. The most common manifestations of *Staphylococcus* infections are swollen joints and feet, this swelling will eventually cause lameness of the chicken and loss of performance (Barnes, 2008).

Escherichia coli

The bacterial genera *Escherichia* is characterized by gram negative, facultatively anaerobic rods, that can be either motile or nonmotile, that will ferment glucose and other carbohydrates (Bergey's Manual 9th edition, 1994). *E. coli* strains are responsible for approximately 200,000 cases of human food-borne illness per year, of these an estimated 7% are from poultry meat products (Scallan *et al.*, 2011; Batz *et al.*, 2011). Dhanarani *et*

al. (2009) observed that, of the total bacterial population in poultry litter, *E. coli* accounted for 12.5%. *E. coli* concentration in poultry litter has been reported between 10^5 and 10^{10} with an average of 10^9 cfu/g of litter (Terzich *et al.*, 2000). These numbers are two orders of magnitude less than those authors reported for *Staphylococcus* numbers of 10^{11} cfu/g. However, a survey of 86 litter samples from broiler houses in Georgia yielded only 2 samples positive for *E. coli* (Martin and McCann, 1998).

In poultry, avian pathogenic *E. coli* causes colibacillosis, this infection presents itself as either air sacculitis, colisepticemia, coligranuloma, coliform cellulitis and other diseases (Barnes, 2008). Performance loss and mortality associated with colibacillosis can be extremely high with almost all flocks being affected, and some of the more virulent strains cause death within a few hours of infection (Barnes, 2008).

Campylobacter spp.

Campylobacter is quickly becoming one of the leading causes of foodborne illnesses in the United States, accounting for approximately 850,000 cases (Scallan *et al.*, 2011). It has been estimated that 72% of the total cases of human food-borne *Campylobacter* are of poultry meat origin (Batz *et al.*, 2011). *Campylobacter* is characterized as being gram negative curved or spiral rod, with cork-screw-like motility by means of a single polar flagellum. In the laboratory, *Campylobacter* spp. grow best at 42° C under microaerophilic (5% O₂, 10% CO₂, 85% N₂) atmospheric gas conditions (Bergey's Manual 9th Edition, 1994).

Litter that has been contaminated with *Campylobacter* has been shown to be capable of colonizing broilers. Thus one contaminated flock can cause colonization of sequential flocks of broilers when using built up litter, or litter that has been left in the

house for several flocks (Montrose *et al.*, 1984). However the detection of *Campylobacter* in broiler litter is infrequent. Utilizing PCR methods, Lu *et al.* (2003) and Roberts *et al.* (2009) found no *Campylobacter*, but Rothrock *et al.* (2008a) found *Campylobacter* in 40% of litter sampled. While performing environmental sampling, which included collecting litter around poultry houses, Bang *et al.* (2002) found only 3.3% of the samples were positive for *Campylobacter* by culture methods, but around 30% of the samples were positive using 16s rRNA gene analysis. This finding led the researchers to believe that *Campylobacter* did not persist long in the environment, or became nonculturable.

***Clostridia* spp.**

Clostridia are another important group of bacteria found in poultry litter, not only for human health, but also for bird health. These bacteria are characterized as being gram positive rods, motile by peritrichous flagella, having a strict anaerobic metabolism and able to produce numerous toxins (Bergey's Manual 9th Edition, 1994). *Clostridia* infections in humans account for nearly 965,000 illnesses a year and it has been estimated that all cases are food-borne in origin (Scallan *et al.*, 2011). In broiler operations, the impact of *C. perfringens* can be severe and costly, *C. perfringens* can cause necrotic enteritis, which leads to morbidity and mortality (Immerseel *et al.*, 2004; Long *et al.*, 1974; Opengart, 2008a). In broiler flocks with high levels of *C. perfringens* performance reduction can range from 25-43%, these losses were attributed to lowered feed conversion, caused by damage to the intestine, which led to reduced weight at slaughter (Lovland and Kaldhusal, 2001). Infection with *C. perfringens* type A can cause gangrenous dermatitis, a disease diagnosed by necrotic lesions of the skin and

subcutaneous tissues. Gangrenous dermatitis is commonly associated with older broilers and can cause sudden mortality, which can lead to severe profit losses depending on the severity of the outbreak (Opengart, 2008b). In 1968, Alexander *et al.* isolated *Clostridia* spp. from 60% of litter samples, and found *C. perfringens* in 8 out of 44 litter samples. Lu *et al.* (2003) found that 7.78% of potentially pathogenic bacteria in litter were some type of *Clostridium* spp., by 16S rRNA sequencing. *C. perfringens* has also been found at detectable levels in litter regardless of sampling area (Roberts *et al.*, 2009).

***Salmonella* spp.**

Salmonella spp. are the leading cause of bacterial human food-borne gastrointestinal illnesses, accounting for more than 1 million cases per year in the United States (Scallan *et al.*, 2011). This bacterium is identified as being gram negative rod, facultatively anaerobic, motile by peritrichous flagella, produces H₂S, facultative intracellular organism and the causative agent of typhoid, gastroenteritis and septicemia (Bergey's Manual 9th Edition, 1994). Among food, contaminated poultry products account for 21% of the total cases, the highest rate among food products (Batz *et al.*, 2011). *Salmonella* however has proven to be elusive in poultry litter; Lu *et al.* (2003) detected no *Salmonella* by culture methods and molecular analysis in used broiler litter. In contrast, *Salmonella* was detected on 83% of farms that reuse litter and 68% of farms that dispose of litter after each flock in a study looking at Australian broiler farms (Chinivisagam *et al.*, 2010). The concentrations of *Salmonella* in each litter sample varied, with almost a 5 log difference between samples, where the researchers attributed this to several factors including transmission from parent flocks and environmental conditions (Chinivisagam *et al.*, 2010). Comparison of soil characteristics and effects on

Salmonella incidence in broiler litter yielded 29% of litter samples being positive (Volkova *et al.*, 2009).

Salmonella is known to possess an acid tolerance response system by which it can survive in acidic environments; this response is based on the production of several acid shock proteins during exposure to low pH environments (Foster and Hall, 1990; Foster, 1993). It involves two stages: the first stage occurs at a pH of 5.8, during this stage the *Salmonella* cells can maintain pH homeostasis down to pH values below 4.0. The second stage is induced during a pH shift to 4.5 or less, this stage involves the production of additional acid shock proteins that allow the bacterium to survive at a pH of 3.3 or less. However the researchers found that these proteins were only produced for short periods of time, approximately 30-40 minutes (Foster, 1993). Other research into this acid tolerance response has shown that virulent strains of *Salmonella* are much more likely to survive low pH environments than avirulent strains. This system would be very important to survive passage into the small intestine through the stomach (Lee *et al.*, 1994).

Distribution of Bacteria in Poultry Litter

Little is known regarding the distribution of bacteria in broiler litter at different locations in a commercial broiler house. Differences in populations were found using denaturing gradient gel electrophoresis at several locations within broiler houses: feed lines, water lines, front (evaporative cooling pads), middle of house and back (exhaust fans). Differences were also found in litter temperature, pH and moisture at each location, with moisture and temperature having the greatest effects on bacteria present

(Lovahn *et al.*, 2007). The researchers concluded that major spatial shifts in microbial populations were possible by moving just a few centimeters.

Reusing broiler litter is common practice, with some being used for years (Vizzier-Thaxton *et al.*, 2003). During this reuse, the amount of litter increases as does the depth of the litter. During buildup, some or all of the litter must be removed by the grower, and most companies require litter depth to be maintained between three and eight inches. Little is known about the microbial make up of the deeper depths of broiler litter, considering most samples are taken from the top portion of the litter (Lu *et al.*, 2003; Dhanarani *et al.*, 2009; Graham *et al.*, 2009; Lovahn *et al.*, 2007; Omeira *et al.*, 2006; Pope and Cherry, 2000; Scheffererle, 1965; Smith *et al.*, 2007; Terzich *et al.*, 2000; Vizzier-Thaxton *et al.*, 2003). These samples were taken by scraping the heel of the hand into the top two inches of litter and placing this sample in a sterile bag, sometimes mixed with samples from other locations in the poultry house. When sampled, the bottom fraction of poultry litter has yielded a different bacterial make up than the top fraction (McCrea *et al.*, 2008, Barker *et al.*, 2010). The examination of the bottom two inches of poultry litter resulted in several different *E. coli* isolates than what was found in the top portion of litter (McCrea *et al.*, 2008). In 2010, Barker *et al.* examined the top, middle and bottom depths of broiler litter. A direct relationship was found between depth of litter and total bacteria present. Bacteria numbers decreased significantly between top, middle, and bottom fractions for total aerobes, anaerobes and coliforms concentrations. The researchers concluded constant shedding of new bacteria by means of fecal material and the abundance of energy in the top fraction of litter led to higher bacterial concentration as compared to the middle and bottom fractions. In poultry houses with

built up litter of many inches, sampling the top few inches of the litter could give a skewed representation of the overall bacterial population. A clearer picture of the microbial population in poultry litter is needed, as it would give more accurate concentrations of the bacteria present in litter. If it is shown that bacteria numbers are significantly less in the bottom fraction of the litter, then perhaps raking or mixing the top fraction with the bottom fraction would dilute the bacteria. By diluting the bacteria, it may be possible to reduce the spread of pathogenic bacteria for poultry and people from one flock to the next.

Litter Management and Effects on Bacteria

Different litter management techniques have been found to be very effective at reducing bacterial numbers. Two of the more common and most effective methods are composting to heat the litter and lowering the litter pH by use of litter amendments.

Composting to heat litter to a temperature adequate to kill bacteria has been shown to be an effective means of reducing bacterial numbers (Macklin *et al.*, 2006; Macklin *et al.*, 2008 Bush *et al.*, 2007; Kelleher *et al.*, 2002). Macklin *et al.* (2006) found that as little as 7 days of in house composting could be effective at reducing bacterial numbers. With only 7 days of composting it was reported that a 1 log reduction in total aerobic bacteria and a 2 log reduction in total anaerobic bacteria numbers were detected compared to controls. The researchers hypothesized that heat as well as possible ammonia buildup were major contributing factors to bacteria reduction.

A significant reduction of pathogens by composting has also been observed. In deep stacked litter, at least 2 meters in thickness, *Salmonella* has been shown to be reduced by a maximum of 98.7% (Bush *et al.*, 2007). Macklin *et al.* (2008) found a

significant reduction in *C. perfringens* in composted litter, as well as complete eradication of viable *Campylobacter* and *Salmonella*. The researchers noted that heat generated by the compost was a major factor in reducing *Salmonella*. In contrast, other research has shown that composting is not effective at reducing pathogens.

Campylobacter has been found to persist in bovine manure compost for up to 120 days (Inglis *et al.*, 2010). However, the researchers noted that most viable *Campylobacter* was recovered very early in composting, 15 days or less. The researchers did find *Campylobacter* DNA in all compost piles tested regardless of treatment

In a survey of bacteria in poultry litter, it was found that samples with lower pH had lower bacterial counts (Terzich *et al.*, 2000). The use of litter amendments to reduce ammonia emissions is a common practice in broiler houses (Terzich *et al.*, 1998). These amendments reduce ammonia by direct chemical interactions, reduction in litter pH and reduced numbers of ammonia producing bacteria (Terzich *et al.*, 1998).

Lowering the litter pH has been shown to be effective at reducing bacterial numbers (Choi *et al.*, 2008; Cook *et al.*, 2006; Cook *et al.*, 2008 Rothrock *et al.*, 2008b; Payne *et al.*, 2007; Pope and Cherry, 2000). Choi *et al.* (2008), using the litter amendments Al_2SO_4 , AlCl_3 and FeSO_4 , found a significant reduction in total aerobes and gram negative bacteria for all three amendments. Aluminum sulfate has been shown to completely eliminate *Campylobacter* from litter (Cook *et al.*, 2006). However, another study with aluminum sulfate showed a significant decrease in *Campylobacter*, a 3 log reduction instead of eradication, as well as a significant decrease in *E. coli*. That study showed an overall reduction in bacterial numbers but not a reduction in bacterial diversity (Rothrock *et al.*, 2008). Sodium bisulfate is another acidifying litter amendment that has

been shown to reduce pH and bacteria. It has been found to reduce pH as low as 1.2 in treated litter, and reduce bacterial numbers 1 – 2 log over untreated litter (Pope and Cherry, 2000). This same study sampled birds in the processing plant as well, and could find no difference between treated and untreated litter on the total bacterial numbers on birds inside the processing plant.

While litter amendments or treatments have shown effects on bacteria levels in general, their use against *Salmonella* is not as effective. Payne *et al.* (2007) found the greatest reduction in *Salmonella* when pH was 4 or less, and no significant reduction at pH of 7 and 9. Litter treated with varying levels of aluminum sulfate (3.63 or 7.26 kg/4.6 m²) or sodium bisulfate (1.13 or 1.81 kg/4.6 m²), which lowered litter pH to less than 5, showed no reduction in *Salmonella* concentrations or frequency for whole carcass rinses or ceca samples (Line, 2002). Commercially available sodium bisulfate applied at the manufacturer's guidelines (2.27 kg /9.29 m²) also yielded no significant effect on *Salmonella* populations in broiler litter, even though a pH of 1.2 was achieved (Pope and Cherry, 2000). Payne *et al.* (2002) used granulated sulfuric acid and sodium bisulfate at various application levels on used litter inoculated with *Salmonella*. High levels of both granulated sulfuric acid and sodium bisulfate (45.4 kg/92.9 m²) were needed to obtain litter pH low enough to significantly reduce *Salmonella* concentration.

Bacillus subtilis

Bacillus subtilis is a bacterium commonly found in soils, it is characterized as a gram positive, obligate aerobe, motile by polar flagella, rod capable of forming endospores (Bergey's Manual 9th edition, 1994). *B. subtilis* has been reported to utilize ammonia as an intermediate for nitrate reduction (Hall and MacVicar, 1955). These

researchers measured nitrate given off by the bacterium from either nitrogen or ammonium source, and discovered that when the initial nitrate supply was depleted that the amount of ammonia used increased dramatically. In 1998, Santoso *et al.* supplemented layer hen and broiler diets with 1 or 2% dried *B. subtilis* culture. It was reported that a significant decrease in ammonia production, inside the house and in stored manure, was observed for broilers and layers. No significant effect was found in broiler body weight gain or feed intake, but feed conversion was decreased by 0.1 lbs feed per lb weight gain with the inclusion of *B. subtilis* in the diet. In addition, a reduction in the weight of excreta produced was observed in broilers receiving *B. subtilis*. The researchers concluded that this would reduce the amount of pollutants from poultry sources.

In an effort to reduce the colonization of broiler type chickens with *S. Enteritidis* and *C. perfringens*, La Ragione and Woodward (2003) inoculated day old chicks with 10^9 *B. subtilis* spores, and once the chicks were two days old either 10^5 *S. Enteritidis* or *C. perfringens* cells. Twenty four hours after challenge, the birds treated with *B. subtilis* had significantly less *Salmonella* and *C. perfringens* recovered from organs than birds receiving only *Salmonella*. However, after the first 24 hours no reduction in *Salmonella* was observed. Of importance to litter management is the shedding of bacteria by birds through excreta onto the litter. In the experiment, shedding of *Salmonella* and *C. perfringens* in feces was monitored for 36 days, and birds receiving *B. subtilis* shed significantly less *Salmonella* and *C. perfringens* than control birds for each sampling time. In agreement, Fritts *et al.* (2000) found that birds fed *B. subtilis* had significantly better feed conversion, lower total bacteria and *E. coli* carcass counts over a 42 day grow-out period. It was also observed that *B. subtilis* reduced the incidence of carcasses

contaminated with *Salmonella*. While all control carcasses were positive for *Salmonella* only half of the treated carcasses tested positive.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was developed in the 1980's by Kerry Mullis as a way to amplify small amounts of DNA (Mullis and Faloona, 1987). Since then, PCR has become a common molecular technique. Small amounts of a target gene or sequence in a longer DNA sequence are amplified using, DNA polymerase, buffer, Mg^{2+} , and oligonucleotide primers that attach to the target sequence. This reaction mix is amplified by changing temperatures to cause several different reactions to occur. First, in a denaturation step the DNA is heated to break the double helix, the mix is then cooled to allow the primers to anneal to the target DNA sequence. The mix is then heated to elongate the sequence to which the primer is attached. The sequence is elongated when polymerase binds to the 3' end of the primer and the appropriate nucleotides are added. The resulting product and original DNA can be cycled numerous times. Cycling causes the DNA to be amplified exponentially, which allows even small amounts of DNA to be studied (Saiki *et al.*, 1985, Saiki *et al.*, 1988).

Taq polymerase, isolated from *Thermus aquaticus*, has the ability to attach all 4 different nucleotides onto the newly formed DNA strand. The bonds between polymerase and nucleotides have been shown to be mostly nonspecific, allowing for the attachment of the different structures present on each nucleotide. The primer and DNA strand fit snugly into a the groove formed by the palm of the polymerase. The "fingers" of the polymerase enzyme are able to rotate this action brings a new nucleotide in close enough proximity with the DNA template for attachment. The Mg^{2+} , added to PCR in

the form of $MgCl_2$, is essential for the catalytic mechanism of the polymerase. The thermostability of the Taq polymerase allows it to remain viable during the high temperatures needed for denaturation of the double stranded DNA during each amplification cycle.

Pyrosequencing

Since the advent of DNA sequencing, the increasing ease of preparing samples for sequencing has driven the need for higher throughput sequencing methods. One such method called pyrosequencing detects individual nucleotides based on the release of pyrophosphate during DNA elongation (Nyren, 1987). Upon release, luciferase is attached to the pyrophosphate by ATP sulfurylase, light emitted by the luciferase is then detected by a luminometer. In 1996, Ronaghi *et al.* greatly improved upon this method by allowing the sequencing reactions to be monitored in real time. The method developed by Ronaghi *et al.* (1996) also successfully attached DNA strands to paramagnetic beads, keeping the strands stable and eliminated the need for electrophoresis gels. Combined, this greatly improves the efficiency and ease with which samples could be sequenced. The drawback to this method is that the sequences must be shorter than those sequenced by the Sanger method, approximately 400-500 base pairs versus 800-1000 respectively.

In recent years, the use of 454 array based pyrosequencing has increased, with the method being used for everything from samples taken from soils to wounds. A survey of soil samples was conducted in four locations in the western hemisphere consisting of three agriculture sites and a forest. It was found that vast differences in the diversity of the two soil types existed. The forest soil had more phyla present than the agriculture

soils, which had many more species from a few phyla (Roesch *et al.*, 2007). Through pyrosequencing, up to 55,000 bacteria 16S rRNA sequences were identified in the soil samples, identifying bacteria from Bacteroidetes, Betaproteobacteria and Alphaproteobacteria as the most abundant bacteria groups present in all soil types sampled. The data obtained also allowed the researchers to identify the relative abundance of each bacteria and the archaea present, which enabled them to compare the effects of land management and soil characteristics on microbial diversity.

In a study of the bacterial community in chronic wounds by both culture and 454 pyrosequencing the diversity of bacterial families was found to be four times higher by pyrosequencing than culture methods (Price *et al.*, 2009). From wounds, 44 bacterial families were identified by 16S rRNA analysis, but only nine of those were found through culture sampling. The results also allowed the researchers to quickly analyze and compare the results from all twenty four patients sampled, which allowed them to notice any differences in bacterial communities that were caused by administration of different antibiotics.

In recent years, to increase the amount of data obtained through pyrosequencing, primers were developed with barcoded tags. Each unique barcoded primer is used to PCR amplify only one sample and can be relatively easily separated during analysis (Parameswaran *et al.*, 2007). A study of the bacterial diversity of cattle feces using such barcoded primers allowed for the sequence analysis of twenty cows, revealing 274 different bacterial species in 142 genera (Dowd *et al.*, 2008). Pyrosequencing results yielded several thousand sequences per sample allowing detection of bacteria that made up less than 0.1% of the total population. The researches concluded that with

pyrosequencing, the bacterial populations of animals can be monitored over time and any shift in the health status of the intestine can be quickly detected.

Molecular analysis of poultry litter

Not all bacteria can be cultured utilizing current growth media. There is substantial difference between the number of cfu's found by culturing and the number of bacteria DNA detected by molecular typing. Not all nutrient requirements, temperatures, atmospheric gas levels can be utilized for culturing. Molecular methods allow for enumeration of bacteria that cannot be cultured without the need for direct counts.

In 2002, Bang *et al.* compared detection frequency of *Campylobacter* in poultry litter using PCR and culture methods. PCR yielded 31% positive samples compared to only 3.3% for culture. The researchers concluded that the PCR allowed for the detection of nonviable, injured or dead bacterial cells. Lu *et al.* (2003) found differences in the bacterial community between PCR and plated methods. Traditional plated culture methods yielded 0.1% Enteric, 13% *Staphylococcus*; while levels detected by 16S rRNA sequencing were 2% Enteric and 8% *Staphylococcus*. Molecular analysis allowed the researchers to identify bacteria that could not be found using media culture methods. Lu *et al.* (2003) also found several bacteria that had never been documented in poultry litter, such as a wood degrading bacteria normally found in the intestines of termites. A novel group of ammonia-producing bacteria was found in poultry litter by PCR detection (Rothrock *et al.*, 2008a). The researchers noted that this group of bacteria had never been isolated in any litter samples by media culture methods.

The PCR technique has pitfalls as well. Lu *et al.* (2003) noted that bacteria present in very small populations will be missed by PCR analysis, due the fact that PCR

amplifies DNA exponentially, therefore the smaller populations will be amplified only slightly while bacterial DNA present in larger amounts will be amplified many times more.

References

- Acosta-Martinez, V. and R. Daren Harmel. 2006. Soil microbial communities and enzyme activities under various poultry litter application rates. *J. Environ. Qual.* 35:1309-1318.
- Alexander, D. C., J.A.J. Carriere, and K. A. McKay. 1968. Bacteriological studies of poultry litter fed to livestock. *Can. Vet. J.* 9(6):127-131.
- Bang, D.D., A. Wedderkopp, K. Pedersen and M. Madsen. 2002. Rapid PCR using nested primers of the 16S rRNA and the hippuricase (*hipO*) genes to detect *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. *Mol. Cell. Probe.* 16:359-369.
- Barker, K. J., J.L. Purswell, J. D. Davis, H. M. Parker, M. T. Kidd, C. D. McDaniel and A. S. Kiess. 2010. Distribution of bacteria at different poultry litter depths. *Int. Poul. Sci.* 9:10-13.
- Barnes, H. J. 2008. Other bacterial diseases. Pages 891-900 in *Diseases of Poultry* 12th edition. Y. M. Saif ed. Blackwell Publishing Professional. Ames, Iowa.
- Batz, M. B., S. Hoffmann and J. G. Morris Jr. 2011. Ranking the risks: The 10 pathogen-food combinations with the greatest burden on public health. Emerging Pathogens Institute, University of Florida.
- Bergey's Manual of Determinative Bacteriology 9th Edition. 1994. John G. Holt ed. Williams and Wilkins, Baltimore.
- Bush, D. J., M. H. Poore, G. M. Rogers and C. Altier. 2007. Effect of stacking method on Salmonella elimination from recycled poultry bedding. *Bioresource Technol.* 98:571-578.
- Chamblee, T. N. and R. L. Todd. 2002. Mississippi broiler litter: fertilizer value and quantity produced. Research Report Mississippi Agricultural and Forestry Experiment Station. Mississippi State University, Starkville, MS. 23(5):1-4.
- Chinivasagam, H. N., M. Redding, G. Runger and P.J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. *Brit. Poultry Sci.* 51:311-318.

- Choi, I. H., J. N. Kim and Y. M. Kwon. 2008. Effects of chemical treatments on pH and bacterial population in poultry litter: a laboratory experiment. *Brit. Poultry Sci.* 49:497-501.
- Cook, K., C. Bolster, M. Rothrock Jr., J. Warren and K. Sistani. 2006. Survival and transport of *Campylobacter jejuni* from poultry litter. ASA-CSSA-SSSa Annual Meeting Abstracts. Indianapolis, IN. Abstract 94-3.
- Cook, K. L., M. J. Rothrock Jr., J. G. Warren, K. R. Sistani and P. A. Moore Jr. 2008. Effect of alum treatment on the concentration of total and ureolytic microorganisms in poultry litter. *J. Environ. Qual.* 37:2360-2367.
- Dhanarani, T. S., C. Shankar, J. Park, M. Dexilin, R. R. Kumar and K. Thamaraiselvi. 2009. Study on acquisition of bacterial antibiotic resistance determinants in poultry litter. *Poult. Sci.* 88:1381-1387.
- Dowd, S. E., T. R. Gallaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort and T. S. Edrington. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rRNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiology.* 8:125-132.
- Foster, J. W. and H. K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella* Typhimurium. *J. Bacteriol.* 172: 771-778.
- Foster, J. H. 1993. The acid tolerance response of *Salmonella* Typhimurium involves transient synthesis of key acid shock proteins. *J. Bacteriol.* 175:1981-1987.
- Fritts, C. A., J. H. Kersey, M. A. Motl, E. C. Kroger, F. Yan, J. Si, Q. Jiang, M. M. Campos, A. L. Waldroup and P. W. Waldroup. 2000. *Bacillus subtilis* C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. *J. Appl. Poult. Res.* 9:149-155.
- Graham, J. P., S. L. Evans, L. B. Price. E. K. Silbergeld. 2009. Fate of antimicrobial-resistant *Enterococci* and *Staphylococci* and resistance determinants in stored poultry litter. *Environ. Res.* 109:682-289.
- Hall, L. M. and R. MacVicar. 1955. Ammonia as an intermediate in nitrate reduction by *Bacillus subtilis*. *J. Bio. Chem.* 213:305-310.
- Immerseel, F. V., J. D. Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck and R. Ducatelle. 2004. *Clostridium perfringens* in poultry: an emerging threat for both animal and public health. *Avian Pathol.* 33(6)537-549.
- Inglis, G. D., T. A. McAllister, F. J. Larney and R. Topp. 2010. Prolonged survival of *Campylobacter* species in bovine manure compost. *Appl. Environ. Microb.* 76(4):1110-1119.

- Khan, A. A., M. S. Nawaz, S. A. Khan and R. Steele. 2002. Detection and characterization of erythromycin-resistant methylase genes in gram-positive bacteria isolated from poultry litter. *Appl. Microbiol. Biot.* 59:377-381.
- Kelleher, B. P., J. J. Leahy, A. M. Henihan, T. F. O'Dwyer, D. Sutton, and M. J. Leahy. 2002. Advances in poultry litter disposal technology – a review. *Bioresource Technol.* 83:27-36.
- Kingery, W. L., C. W. Wood, D. P. Delaney, J. C. Williams and G. L. Mullins. 1994. Impact of long-term land application of broiler litter on environmentally related soil properties. *J. Environ. Qual.* 23:139-147.
- La Ragione, R. M. and M. J. Woodward. 2003. Competitive exclusions by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Vet. Microbiol.* 94:245-256.
- Lee, I. S., J. L. Slonczewski, and J. W. Foster. 1994. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella* Typhimurium. *J. Bacteriol.* 176:1422-1426.
- Line, J. E. 2002. *Campylobacter* and *Salmonella* populations associated with chickens raised on acidified litter. *Poult. Sci.* 81:1473-1477.
- Long, J. R., J. R. Pettit and D. A. Barnum. 1974. Necrotic enteritis in broiler chickens II. Pathology and proposed pathogenesis. *Can. J. Comp. Med.* 38:467-474.
- Lopez-Mosquera, M. E., F. Cabaleiro, M. J. Sainz, A. Lopez-Fabal and E. Carral. 2008. Fertilizing value of broiler litter: Effects of drying and pelletizing. *Bioresource Technol.* 99:5626-5633.
- Lovahn, N., K. L. Cook, M. J. Rothrock, D. M. Miles and K. Sistani. 2007. Spatial shifts in microbial population structure within poultry litter associated with physicochemical properties. *Poult. Sci.* 86:1840-1849.
- Lovland, A. and M. Kaldhusdal. 2001. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated Hepatitis. *Avian Pathol.* 30(1):73-81.
- Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16s rRNA and functional gene markers. *Appl. Environ. Microb.* 69(2):901-908.
- Macklin, K. S., J. B. Hess, S. F. Bilgili and R. A. Norton. 2005. Bacterial levels of pine shavings and sand used as poultry litter. *J. Appl. Poult. Res.* 14:238-245.

- Macklin, K. S., J. B. Hess, S. F. Bilgili and R. A. Norton. 2006. Effects of in-house composting of litter on bacterial levels. *J. Appl. Poult. Res.* 15:531-537.
- Macklin, K. S., J. B. Hess and S. F. Bilgili. 2008. In-house windrow composting and its effects on foodborne pathogens. *J. Appl. Poult. Res.* 17:121-127.
- Martin, S. A. and M. A. Mccann. 1998. Microbiological survey of Georgia poultry litter. *J. Appl. Poult. Res.* 7:90-98.
- McCrea, B. A., K. S. Macklin, R. A. Norton, J. B. Hess and S. F. Bilgili. 2008. Recovery and genetic diversity of *Escherichia coli* isolated from deep litter, shallow litter and surgical shoe covers. *J. Appl. Poult. Res.* 17:237-242.
- Montrose, M. S., S. M. Shane and K. S. Harrington. 1984. Role of litter in the transmission of *Campylobacter jejuni*. *Avian Dis.* 29:392-399.
- Mullis, K. B. and F. A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Method. Enzymol.* 155:335-350.
- Nyren, P. 1987. Enzymatic method for continuous monitoring of DNA polymerase activity. *Anal. Biochem.* 167:235-238
- Omeira, N., E. K. Barbour, P. A. Nehme, S. K. Hamadeh, R. Zurayk and I. Bashour. 2006. Microbiological and chemical properties of litter from different chicken types and production systems. *Sci. Total Environ.* 367:156-162.
- Opengart, K. 2008a. Necrotic enteritis. Pages 872-879 in *Diseases of Poultry 12th Edition*. Y. M. Saif ed. Blackwell Publishing Professional. Ames, Iowa.
- Opengart, K. 2008b. Gangrenous dermatitis. Pages 885-889 in *Diseases of Poultry 12th Edition*. Y. M. Saif ed. Blackwell Publishing Professional. Ames, Iowa.
- Patterson, P. H., E. S. Lorenz and W. D. Weaver, Jr. 1998. Litter production and nutrients from commercial broiler chickens. *J. Appl. Poult. Res.* 7:247-252.
- Parameswaran, P., R. Jalili, L. Tao, S. Shokralla, B. Gharizadeh, M. Ronaghi and A. Z. Fire. 2007. A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing. *Nucleic Acids Res.* 35:1-9.
- J. B. Payne, E. C. Kroger and S. E. Watkins. 2002. Evaluation of litter treatments on *Salmonella* recovery from poultry litter. *J. Appl. Poult. Res.* 11:239-243.
- Payne, J. B., J. A. Osborne, P. K. Jenkins and B. W. Sheldon. 2007. Modeling the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and water activity. *Poult. Sci.* 86:191-201.

- Price, L. B., C. M. Liu, J. H. Melendez, Y. M. Frankel, D. Engelthaler, M. Aziz, J. Bowers, R. Rattray, J. Ravel, C. Kingsley, P. S. Keim, G. S. Lazarus and J. M. Zenilman. 2009. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS One*. 4:1-10.
- Pope, M. J. and T. E. Cherry. 2000. An evaluation of the presence of pathogens on broilers raised on Poultry Litter Treatment®-treated litter. *Poult. Sci.* 79:1351-1355.
- Roberts, B., M. McLaughlin and J. Brooks. 2009. Spatial and temporal analysis of microbial populations in production broiler house litter. American Society of Microbiology. Philadelphia, PA. Meeting Abstract Q-099.
- Roesch, L. F. W., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *International Society for Microbial Ecology Journal*. 1:283-290.
- Ronaghi, M., A. Karamohamed, B. Petterson, M. Uhlen and Pal Nyren. 1996. Realtime DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242:84-89.
- Rothrock Jr., M. J., K. Cook, C. Bolster and J. Sorrell. 2008a. Comparison of molecular methods and traditional plate counting for detecting *Campylobacter jejuni* and *Escherichia Coli* from environmental samples. American Society for Microbiology Annual Meeting. Boston, MA.
- Rothrock Jr., M. J., K. L. Cook, J. G. Warren and K. Sistani. 2008b. The effect of alum addition on microbial communities in poultry litter. *Poult. Sci.* 87:1493-1503.
- Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*. 230:1350-1354.
- Saiki, R. K., D. H. Gelfhand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 239:487-491.
- Santoso, U. S. Ohtani, K. Tanaka and M. Sakaida. 1998. Dried *Bacillus subtilis* culture reduced ammonia gas release in poultry house. *J. Anim. Sci.* 806-809.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis.* 17:7-15.

- Schefferle, H. E. 1965. The microbiology of built up poultry litter. *J. Appl. Bacteriol.* 28(3):403-411.
- Smith, J. L., D. J. V. Drum, Y. Dai, J. M. Kim, S. Sanchez, J. J. Maurer, C. L. Hofacre and M. D. Lee. 2007. Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. *Appl Environ. Microb.* 73(5):1404-1414.
- Stephenson, A. H., T. A. McCaskey and B. G. Ruffin. 1990. A survey of broiler litter composition and potential as a nutrient resource. *Biol. Waste.* 34:1-9.
- Terzich, M., C. Quarles, M. A. Goodwin and J. Brown. 1998. Effect of Poultry Litter Treatment® (PLT®) on the development of respiratory tract lesions in broiler. *Avian Pathol.* 27:566-569.
- Terzich, M., M. J. Pope, T. E. Cherry and J. Hollinger. 2000. Survey of pathogens in poultry litter in the United States. *J. Appl. Poult. Sci.* 9:287-291.
- Vest, L., B. Merka and W. I. Segars. 1994. Poultry waste: Georgia's 50 million dollar forgotten crop. Georgia Cooperative Extension Service Publication. Athens, GA.
- Vizzier-Thaxton, Y., C. L. Balzi and J. D. Tankson. 2003. Relationship of broiler flock numbers to litter microflora. *J. Appl. Poult. Res.* 12:81-84.
- Volkova, V. V., R. H. Bailey and R. W. Willis. 2009. *Salmonella* in broiler litter and properties of soil at farm location. *PLoS ONE.* 4:6403.

Stratification of bacterial concentrations, from upper to lower, in broiler litter

Abstract

There have been numerous research studies investigating the different types of bacteria in broiler litter. However, very little is known about the spatial distribution of bacteria from the top to the bottom of the litter bed. An initial study was performed at the Auburn University Poultry Farm; in which the concentration of bacteria was investigated in floor pens. Core litter samples were taken from 6 individual floor pens, one week prior to placement, day of placement and then at 7, 14, 21, 35 and 49 days of age. From the differences observed in this initial study a larger long term study was deemed necessary. This study involved following bacterial numbers in litter in two commercial broiler houses over the course of eight grow-outs, which was approximately one year. Litter sample cores were taken from each grow-out house, at 14 and 35 days of bird age. Samples were collected near the evaporative cooling pads, middle of the house (control room), and exhaust fans. Samples for analysis were removed from the very upper fraction of litter, the middle of the litter, and the litter directly on top of the dirt pad, being sure to include some dirt pad. The litter was analyzed for: *Staphylococcus* spp., *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., total aerobic, and total anaerobic. A decrease was observed from top to middle and from middle to bottom fractions of litter for all of the bacteria analyses: aerobes ($P < 0.001$), anaerobes ($P < 0.001$), *E. coli* ($P < 0.001$), *Staphylococcus* spp. ($P < 0.001$), *Clostridium perfringens* ($P < 0.001$). In these studies *Salmonella* was not recovered from any of the core litter samples. From upper to lower, all bacteria were reduced by at least 99%. Data indicated that the concentration of bacterial populations within broiler litter could shift dramatically

within only a few inches of litter, and that the lower fraction of litter holds relatively less bacteria than the upper or middle fractions.

Introduction

Broiler litter contains high levels of bacteria, from 10^8 to 10^{11} cfu/g litter (Scheffererle, 1965; Terzich *et al.*, 2000; Macklin *et al.*, 2005; Lu *et al.*, 2003). Common potentially pathogenic bacteria found in broiler litter are: *E. coli*, *Staphylococcus* spp., and *Clostridia* spp., less common are *Salmonella* spp. (Lu *et al.*, 2003; Alexander *et al.*, 1968; Bang *et al.*, 2002; Terzich *et al.*, 2000; Vizzier-Thaxton *et al.*, 2003). *Staphylococcus* spp. has been found to occur in the highest concentrations, and represents the highest percentage of total bacterial population, which can be as high as 29.1% of the total bacteria present within the litter (Terzich *et al.*, 2000; Lu *et al.*, 2003; Omeira *et al.*, 2006; Dhanarani *et al.*, 2009). *E. coli* is commonly used as an indicator of fecal contamination, and is commonly found in broiler litter in significant populations, between 10^5 and 10^{10} cfu/g litter (Terzich *et al.*, 2000).

Salmonella, *E. coli*, *C. perfringens* and *Staphylococcus* are all known to cause significant numbers of food-borne illnesses in people (Scallan *et al.*, 2011). *Salmonella enterica* strains are the leading cause of bacterial food-borne illness in the United States causing more than 1 million cases per year (Scallan *et al.*, 2011). Of these cases approximately 221,000 are of poultry type meat origin (Batz *et al.*, 2011). *E. coli* strains account for approximately 200,000 cases of human food-borne illnesses per year, an estimated seven percent are of poultry meat origin (Scallan *et al.*, 2011; Batz *et al.*, 2011). *C. perfringens* causes 965,000 causes of human food borne illnesses a year, and

Staphylococcus accounts for approximately 240,000 cases (Scallan *et al.*, 2011). Of these bacteria, three can cause illness in poultry, *E.coli*, *C. perfringens* and *Staphylococcus*. *C. perfringens*, can cause necrotic enteritis and gangrenous dermatitis in broilers, both illnesses result in morbidity and or mortality, which can lead to a significant performance loss (Lovland and Kaldhusal, 2001; Opengart 2008). *E. coli*, can cause colibacillosis, this disease isn't typically as severe as diseases caused by *C. perfringens*; however it does lead to live performance reduction.

Staphylococcus spp. are ubiquitous in the environment, and are found in high numbers in poultry litter (Omeira *et al.*, 2006; Terzich *et al.*, 2000). This bacterium is known to cause cellulitis and swollen hocks in poultry, both of which can lead to either impaired growth or a downgrade in the final product (Andreasen, 2008). *Staphylococcus* spp. are noted as being harborers of numerous antibiotic resistance genes that they are capable of being passed along to other bacteria (Khan *et al.*, 2002; Graham *et al.*, 2009). The bacteria tested are all of great importance, either financially to the poultry industry or from a human health standpoint. Information is needed on the distribution of these bacteria within broiler litter in order to effectively control them and to prevent them from entering the bird and ultimately the food chain.

Previous research into litter bacterial levels was conducted by the grab sampling method, which typically examines only the top two inches of litter. The grab sampling method is conducted by scraping the heel of a gloved hand across the top of the litter; this litter is then placed in a bag and can possibly be mixed with litter from other areas (Lu *et al.*, 2003; Terzich *et al.*, 2000; Vizzier-Thaxton *et al.*, 2003). To date, little research has been performed that examined the remaining portion of litter. Research that has been

conducted on the middle and bottom fractions of litter has offered some interesting findings. Litter samples from the bottom two inches of litter have yielded different *E. coli* isolates than the top portion of litter (McCrea *et al.*, 2008). An examination of total aerobes, anaerobes and coliforms yielded a direct relationship between litter depth and bacterial concentration, the researchers found that in the middle and lower fractions of broiler litter bacterial numbers decreased significantly (Barker *et al.*, 2010).

This project aims to find the spatial differences in bacterial concentrations of total bacteria, and a few select pathogenic bacteria of importance to the poultry industry, in three fractions of litter; upper, middle and lower. Initially these differences were measured at the Auburn University Poultry Science Research Unit. A larger, yearlong study was also performed which these parameters were measured on two commercial broiler houses that had clean bedding added at the initiation of the trial.

Materials and Methods

Auburn University Poultry Research Farm

Core litter samples were taken during a single, 49 day broiler grow-out on the Auburn University Poultry Science Research Unit. Each core litter sample was taken from one of six individual floor pens. Samples were taken one week prior to chick placement, day of placement, and then at 7, 14, 21, 35 and 49 days of age. Each pen had litter that was previously used for at least one flock, a nipple watering system and a single gravity type feeder.

Commercial Broiler Farm

Litter samples were taken from a single broiler farm over the course of one year, for a total of eight consecutive grow-outs, starting July 10, 2010 and ending July 27, 2011. This farm was located in northeast Alabama, and consisted of four 50' x 500' and two 60' x 600' solid sidewall houses. The 50' x 500' houses were 7 years old and the 60' x 600' houses were new, with the first flock being placed in July 2010. At the initiation of the study new pine shaving litter was added to the houses to be sampled.

Litter samples were taken twice per grow-out flock at 14 and 35 days of age. Core litter samples were taken from, one 50' x 500' house and one 60' x 600' foot house. Cores were taken in the open area in the middle of the house near the evaporative cooling cells, center of house (near control room) and exhaust fans (Figure 1).

Litter Sampling and Microbiological Methods

Core litter samples were taken using one of six sterile 5.08 cm diameter PVC pipes. Each PVC pipe had a 2.54 cm x 25.4 cm window cut into the side to allow for litter removal, these windows were sealed with duct tape during sample harvesting. Measurement markings were made on the side of the window so that litter depth could be determined, this would serve later as a guide when removing litter. Pipes were driven down through the litter and into the hard dirt pad underneath, which would serve to plug the bottom of the pipe, so litter would not fall out. After each sample had been taken, the PVC pipe was sealed on top and bottom with duct tape for transport to the laboratory.

From each core sample, 10 g of litter was taken from upper, middle and lower and placed into whirl pak bags. Top litter was considered that which was directly on top of

the core, middle was litter halfway down the core and bottom was litter directly above and including the hard dirt pad. To each sample, 90 mL of Phosphate Buffered Saline (PBS, Difco™ Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was added, and then homogenized for 1 minute. Dilutions (1:10) were performed in tubes containing 9 ml PBS until sufficient dilution was achieved.

From the dilution tubes, 100 μ L was spread plated onto duplicate plates of differential media. Three dilutions per sample were plated onto the following media: aerobes, plate count agar (Difco™); anaerobes, anaerobic agar (Difco™); *C. perfringens*, Oxoid Tryptose sulfite cycloserine agar (Oxoid); *E. coli*, MacConkey agar (Difco™); *Staphylococcus*, Baird Parker agar with tellurite (Accumeida, Neogen USA); *Salmonella*, Xylose-Lysine-Tergitol 4 agar (Difco™). *Salmonella* was enriched in Tetrathionate broth, Hajna (Difco™) before plating. Total anaerobes and *C. perfringens* plates were incubated in an anaerobic chamber at 37° C in 5% H₂, 5% CO₂ and 90% N₂ for 24 hours. All other plates were incubated at 37° C in an aerobic atmosphere for 24 hours. An exception was *Staphylococcus* plates which were incubated for 48 hours to allow colonies to grow larger. After incubation, plates were removed from the incubator and enumerated.

Data were analyzed in SAS 9.2, using General Linear Models function at $P \leq 0.05$, for effects due to bird age, flock number, house, location within house, litter depth and interactions (SAS Institute, 2009). Significant differences were separated by either T test (bird age) or Tukey's Honestly Significant Difference Test. Bacterial counts were log₁₀ transformed before analysis. Results given are composite data from all litter samples taken.

Results

Auburn University Poultry Science Research Unit

Bacteria in each fraction

As shown in Table 1, all bacteria enumerated decrease in concentration from the upper to middle and lower fractions of litter. For all bacteria tested, except *C. perfringens*, there was an approximate two log or 99% decrease between the upper and lower litter fractions.

A decrease in total aerobic bacteria was seen between the upper fraction and both middle and lower fractions, but this decrease was not observed between the middle and lower fractions ($P=0.01$).

Total anaerobic bacteria were observed to have the largest decrease in concentration. From upper to middle there was a 1.4 log reduction. From middle to lower there was a 1.2 log reduction and overall (upper to lower) there was a 2.6 log reduction.

Staphylococcus concentration was reduced by 1.1 log from upper to middle and 1.0 log from middle to lower. Observed *Staphylococcus* numbers were actually higher in the upper fraction of the litter than total aerobic bacteria and equal in number in the middle fraction. This implies that either the majority of aerobic bacteria are *Staphylococcus* or that the medium utilized grew other bacteria.

E. coli accounted for the largest reduction in bacteria from the upper to middle fraction, 1.7 logs. Despite this high reduction, less than a half log decrease in *E. coli* was

observed between the middle and lower fractions. *C. perfringens* was recovered in low concentrations in the upper and middle fractions and not recovered in the lower fraction.

Bacteria concentrations by bird age

Increasing numbers of bacteria were observed as birds aged (Table 2), as much as a 5 log increase in the case of *E. coli*. Despite the trend of increasing bacteria, total aerobic bacteria increased only numerically, rising only seven tenths of a log before placement to its peak at 21 days ($P=0.514$). Total anaerobic bacteria increased significantly, by 3.6 logs, with lowest numbers observed prior to placement and peaking at 21 days of age ($P<0.001$). After peaking, total anaerobic bacteria decreased in number until the end of the study at 49 days.

Staphylococcus increased by several orders of magnitude from chick placement to peak at 21 days of age. *Staphylococcus* numbers did not decrease, unlike the rest of the bacteria studied.

The highest increase in bacteria concentration was found in *E. coli*. *E. coli* concentration started at 0.4 log₁₀ cfu/g litter, when chicks were 1 day of age, then increased by 5 logs to peak at 14 days of age at 5.4 log₁₀ cfu/g litter. The number of *E. coli* cells remained steady until 35 days, when a decline started and continued until the conclusion of the grow-out at 49 days.

C. perfringens, like the other bacteria, increased in concentration until 21 days of bird age and then numbers decreased, but these changes were not statistical ($P=0.156$).

Commercial broiler farm

As can be seen in Figure 2, there was a significant decrease in the middle and lower fractions of poultry litter for total aerobic bacteria from samples taken at a commercial broiler farm. Results for the middle and lower fractions indicate aerobes occurring at 7.72 and 6.14 \log_{10} cfu/g litter respectively, concentration in the upper fraction was 8.58 \log_{10} cfu/g ($P < 0.001$).

Unexpectedly, the number of anaerobic bacteria did not increase in the lower fraction of litter (Figure 3). The upper had 6.1 \log_{10} cfu/g, middle 4.8 and lower 2.3. The results indicate a 4 log decrease from the upper to the lower fraction.

The mean concentrations of *Staphylococcus* found in each litter fraction are shown in Figure 4. *Staphylococcus spp.*, were highest in the upper fraction (8.5 \log_{10} cfu/g litter) and decreased significantly with each lower fraction (7.3 and 5.7 \log_{10} cfu/g litter) ($P < 0.001$). The data shows an overall 99.9% decrease in the concentration of *Staphylococcus* from upper to lower.

Figure 5 displays the decrease in *E. coli* concentration from upper to lower. *E. coli* concentration in the upper fraction was 4.4 \log_{10} cfu/g and is almost undetectable with less than 1 \log_{10} cfu/g in the lower fraction ($P < 0.001$). The middle fraction had 1.8 \log_{10} cfu/g litter of *E. coli*.

C. perfringens, while not present in high numbers, was still found in all three fractions of litter as shown in Figure 6. In the upper and middle fractions, *C. perfringens* was recovered at an average concentration of 1.6 and 1.0 \log_{10} cfu/g litter. In the lower fraction *C. perfringens* was recovered at less than a tenth of a log per gram.

In Table 3, differences in mean concentrations for each sampling type are shown. The only statistical difference is the higher concentration of total aerobes found at the middle of the house than at the evaporative cooling cells ($P < 0.001$). No *Salmonella* was recovered in either study (Data not shown).

Discussion

The multiple \log_{10} cfu/g litter decrease in all bacteria types measured between upper, middle, and lower fractions of litter show that in just a few inches of litter the overall bacterial load can decrease by 99% or more. These findings are in agreement with Barker *et al.* (2010) who found a significant decrease in total aerobes, anaerobes and coliform bacteria between upper and lower fractions of litter. This observation was expected as the upper fraction of litter receives a constant supply of new bacteria from excreta, in addition it receives ample water, nutrients and oxygen. The most significant decrease was observed in the lower fraction of litter, which contained a portion of the dirt pad. Previous studies have shown litter bacterial loads up to 10^{11} cfu/g litter, by the grab sampling method at various locations within the house including, but not limited to; feed and water lines, side walls, evaporative cooling cells and exhaust fans. The researchers would then extrapolate those results to the entire quantity of litter in the house (Scheffererle, 1965; Terzich *et al.*, 2000; Macklin *et al.*, 2005; Lu *et al.*, 2003).

Data in this paper implies that grab sample findings may inadequately describe the litter bacteria community, simply because the bacteria are not evenly distributed, and that beyond the upper 2 inches of litter bacterial numbers are significantly reduced by several orders of magnitude. Bacterial concentrations being lower in this paper than

reported in previous research could also be attributed to the small sampling area, a larger sampling area in the case of grab sampling would allow for areas of higher bacterial populations to be added to the samples, i.e. water lines, feed lines, or any other areas with higher bird density. As core samples were taken in the center of the house, this area would have less bird density and therefore fewer bacteria. It was hypothesized that the lower depth of litter would have reduced oxygen content and therefore yield a more anaerobic environment. However, data shown here disputes that hypothesis as less than 3 \log_{10} cfu/g litter were found in the lower fraction of litter. This could also be attributed to sampling technique, as strict anaerobic conditions were not kept during sampling and true anaerobes would be killed leaving only spore forming and facultative anaerobes. The major contributing factor to the higher bacterial concentrations in upper fraction of litter is simply the constant shedding of feces onto the surface of the litter. Bacteria either do not survive or do not progress into middle and lower fractions of litter.

The increase observed in bacteria concentrations over the flock age is in agreement with Macklin *et al.* (2005). Before chick placement, the researchers observed total aerobic bacteria levels of 10^5 cfu/g of litter, which then increased to 10^8 immediately after bird placement, and reached their highest concentration during week 4 at 10^{10} cfu/g, followed by a subsequent decline. This observation shows that once birds are placed and begin shedding bacteria through fecal material onto the litter, the number of bacteria rapidly increases, but will level off around three to four weeks of age. Once the upper limit for bacteria has been reached, the decline suggests that the litter can only support a certain number of bacteria, despite the constant addition of new bacteria.

Total aerobic bacteria concentration being higher in the middle sampling location can be attributed to center house brooding. These broilers were raised strictly in the center of the house for the first seven to ten days, and then moved into the remainder of house. This early, concentrated deposition of bacteria could lead to this area having higher numbers of bacteria.

Significantly, lower bacterial numbers in the lower two thirds of the litter bed show that extrapolating the entire litter bacterial population from samples of the upper two inches of litter is not adequate to describe the entire litter bacterial community. This data implies that a removal of the upper portion of the litter may be sufficient to reduce bacterial numbers in broiler houses, where litter is used for several flocks, to lower levels. Putting less or possibly no new litter into the house could save the grower a substantial amount of money.

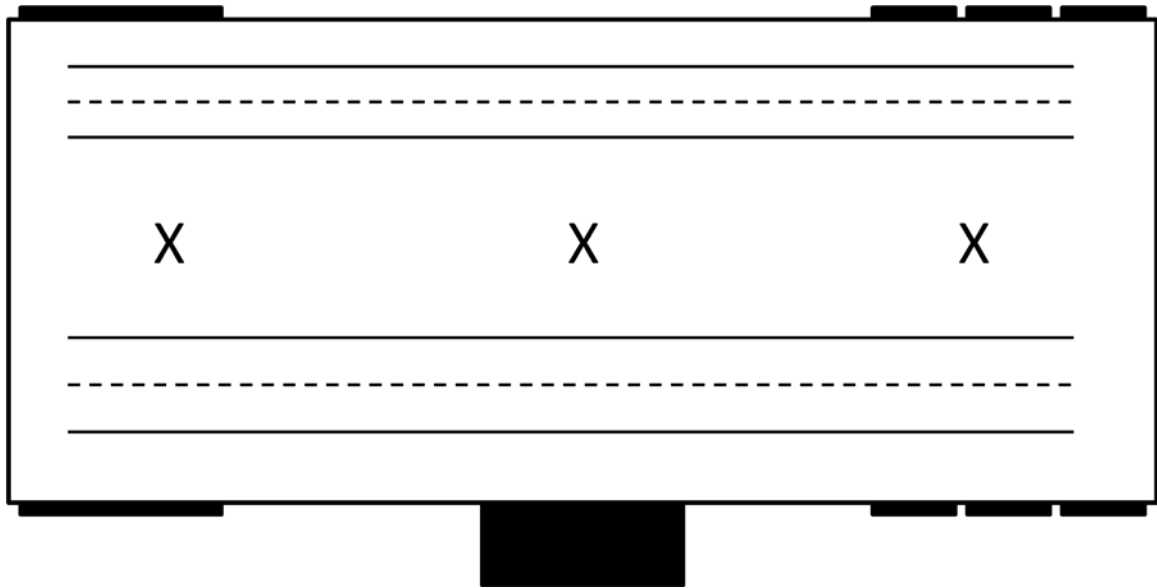
References

- Alexander, D. C., J.A.J. Carriere, and K. A. McKay. 1968. Bacteriological studies of poultry litter fed to livestock. *Can. Vet. J.* 9(6):127-131.
- Andreasen, C. B. 2008. Staphylococcosis. Pages 892-900 in *Diseases of Poultry* 12th Edition. Y. M. Saif ed. Blackwell Publishing Professional. Ames, Iowa.
- Bang, D.D., A. Wedderkopp, K. Pedersen and M. Madsen. 2002. Rapid PCR using nested primers of the 16S rRNA and the hippuricase (hipO) genes to detect *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. *Mol. Cell. Probe.* 16:359-369.
- Barker, K. J., J.L. Purswell, J. D. Davis, H. M. Parker, M. T. Kidd, C. D. McDaniel and A. S. Kiess. 2010. Distribution of bacteria at different poultry litter depths. *Int. J. Poult. Sci.* 9:10-13.
- Batz, M. B., S. Hoffmann and J. G. Morris Jr. 2011. Ranking the risks: The 10 pathogen-food combinations with the greatest burden on public health. Emerging Pathogens Institute, University of Florida.

- Dhanarani, T. S., C. Shankar, J. Park, M. Dexilin, R. R. Kumar and K. Thamaraiselvi. 2009. Study on acquisition of bacterial antibiotic resistance determinants in poultry litter. *Poult. Sci.* 88:1381-1387.
- Graham, J. P., S. L. Evans, L. B. Price, E. K. Silbergeld. 2009. Fate of antimicrobial-resistant enterococci and staphylococci and resistance determinants in stored poultry litter. *Environ. Res.* 109:682-289.
- Khan, A. A., M. S. Nawaz, S. A. Khan and R. Steele. 2002. Detection and characterization of erythromycin-resistant methylase genes in gram-positive bacteria isolated from poultry litter. *Appl. Microbiol. Biot.* 59:377-381.
- Lovland, A. and M. Kaldhusdal. 2001. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated Hepatitis. *Avian Pathol.* 30(1):73-81.
- Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16s rRNA and functional gene markers. *Appl. Environ. Microb.* 69(2):901-908.
- Macklin, K. S., J. B. Hess, S. F. Bilgili and R. A. Norton. 2005. Bacterial levels of pine shavings and sand used as poultry litter. *J. Appl. Poult. Res.* 14:238-245.
- McCrea, B. A., K. S. Macklin, R. A. Norton, J. B. Hess and S. F. Bilgili. 2008. Recovery and genetic diversity of *Escherichia coli* isolated from deep litter, shallow litter and surgical shoe covers. *J. Appl. Poult. Res.* 17:237-242.
- Omeira, N., E. K. Barbour, P. A. Nehme, S. K. Hamadeh, R. Zurayk and I. Bashour. 2006. Microbiological and chemical properties of litter from different chicken types and production systems. *Sci. Total Environ.* 367:156-162.
- Opengart, K. 2008. Necrotic Enteritis. Pages 872-879 in *Disease of Poultry* 12th edition. Y. M. Saif ed.
- SAS Institute. 2009. SAS User's Guide. Statistics. Version 9.2 Edition. SAS Inst. Inc, Cary, NC.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7-15.
- Schefferle, H. E. 1965. The microbiology of built up poultry litter. *J. Appl. Bacteriol.* 28(3):403-411.

Terzich, M., M. J. Pope, T. E. Cherry and J. Hollinger. 2000. Survey of pathogens in poultry litter in the United States. *J. Appl. Poult. Sci.* 9:287-291.

Vizzier-Thaxton, Y., C. L. Balzi and J. D. Tankson. 2003. Relationship of broiler flock numbers to litter microflora. *J. Appl. Poult. Res.* 12:81-84.



Evaporative Cooling Cells

Control Room

Exhaust Fans

Figure 1. Graphical representation of a generic commercial broiler house, evaporative cooling cells on the left, control room in middle, and exhaust fans on the right, solid lines represent water lines, and hatched lines represent feed lines. Each “X” marks approximate sampling location where core litter samples were taken, dashed represent feed lines, while solid lines represent water lines.

Table 1. Composite litter fraction results for preliminary Auburn University Science Research Unit (Log₁₀ cfu/g litter)

Litter Fraction	Bacterium				
	Aerobic	Anaerobic	<i>Staphylococcus</i> spp.	<i>E. coli</i>	<i>C. perfringens</i>
Upper	8.6 ^x	7.1 ^x	8.7 ^x	4.4 ^x	0.4 ^y
Middle	7.6 ^y	5.7 ^y	7.6 ^y	2.7 ^y	0.1 ^{yz}
Lower	6.8 ^z	4.5 ^z	6.6 ^z	2.3 ^z	0.0 ^z
SEM	0.313	0.339	.074	0.036	0.121
<i>P</i> value	0.010	<0.001	0.008	0.001	0.044

As can be observed there was a decrease in all bacteria enumerated between all three litter fractions, with the upper having the highest concentration followed by the middle and then lower.

^{x-z} Numbers in a column with different superscripts are different ($P < 0.05$).

SEM = Standard error of mean

Table 2. Bacteria concentrations during a 49-day broiler grow out (Log₁₀ cfu/g litter)

Bird Age (Days)	Bacterium				
	Aerobic	Anaerobic	<i>Staphylococcus</i> spp.	<i>E. coli</i>	<i>C. perfringens</i>
-7 ^a	7.7	3.5 ^z	5.7 ^{yz}	1.2 ^y	0
1	7.2	5.0 ^{xyz}	5.1 ^z	0.4 ^z	0
7	7.4	6.7 ^{xy}	7.2 ^{xy}	3.7 ^w	0.2
14	6.9	6.4 ^{xy}	7.6 ^x	5.4 ^v	0.1
21	8.4	7.1 ^x	8.5 ^x	5.5 ^v	0.6
35	8.2	5.9 ^{xyz}	8.5 ^x	3.6 ^w	0
49	7.8	4.7 ^{xyz}	8.3 ^x	1.9 ^x	0.3
SEM	0.5	0.5	0.1	0.1	0.2
<i>P</i> value	0.514	<0.001	0.015	<0.001	0.156

Data indicates an increase in the number of bacteria present in poultry litter with increasing bird age, peaking between 21 and 35 days. Highest concentrations of anaerobic bacteria, *Staphylococcus* and *E. coli* were observed during these two sampling times. A numerical increase was seen in aerobic and *C. perfringens* concentrations, but this increase was not statistical.

^{w-z}Numbers in a column with different superscripts are significantly different at $P \leq 0.05$

SEM = Standard error of mean

^a -7 = seven days prior to placement

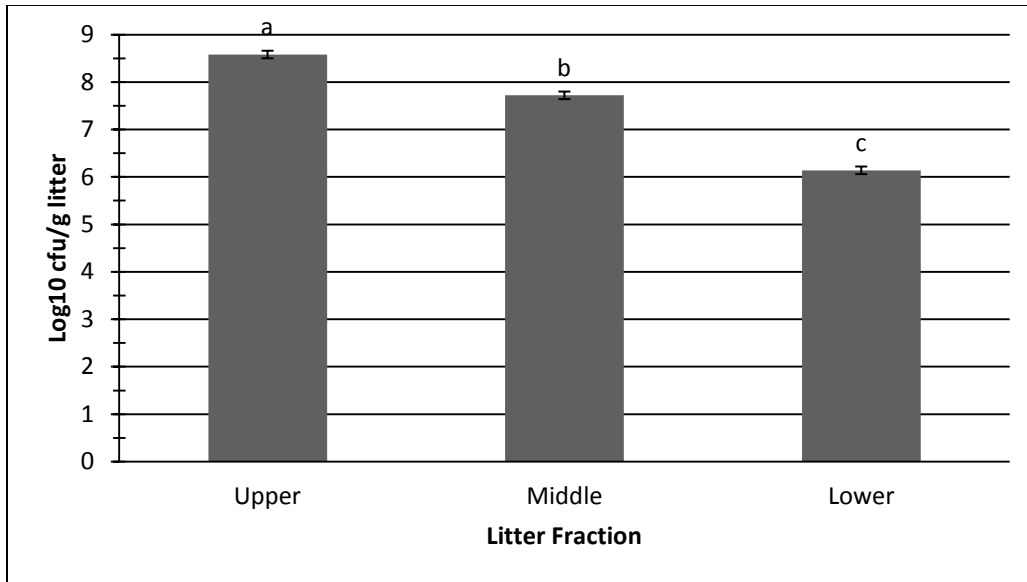


Figure 2. Decrease between upper, middle and lower fraction of litter for total aerobic bacteria. Data is composited from all sampling times. There is a difference between all three fractions, with upper having highest concentration, and lower having the lowest ($P < 0.001$). The total decrease in aerobic bacteria is approximately 2.5 log decrease, representing more than a 99% decrease in total aerobes.

^{a,b,c} Numbers with different superscripts are different ($P < 0.0001$).

Error bars represent the standard error of the mean, ± 0.08

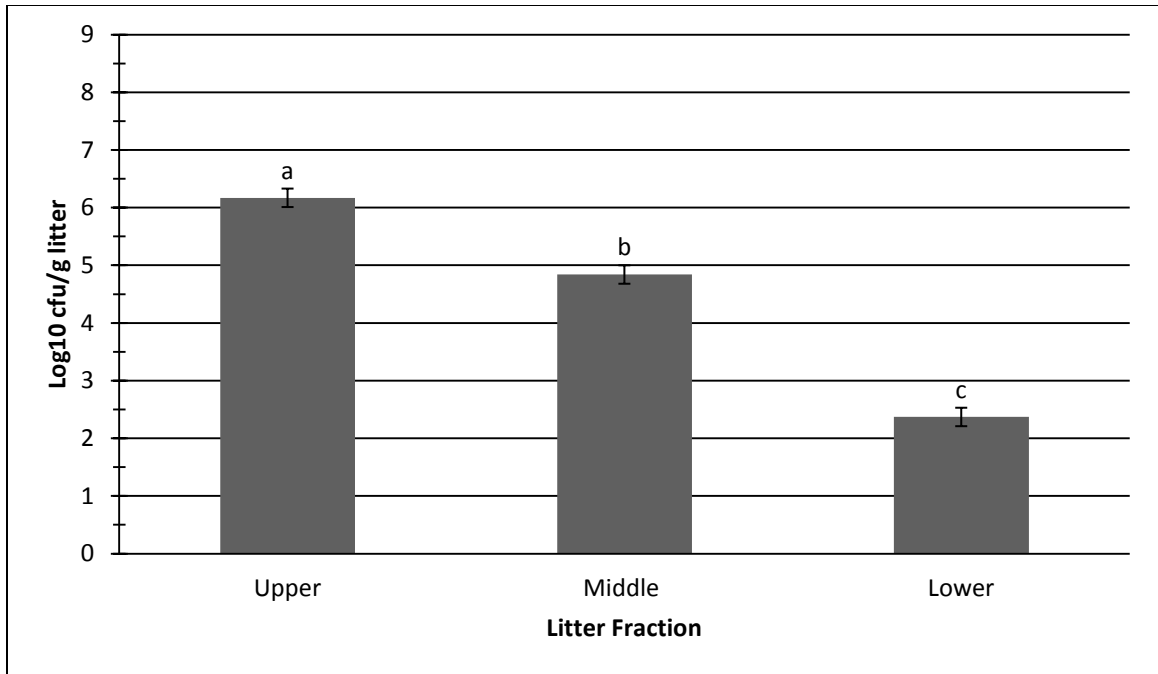


Figure 3. Differences between upper, middle and lower fraction of litter for total anaerobic bacteria. Data given is composited from all samples taken. Differences were observed between all three fractions of litter, with upper fraction having highest concentration of anaerobes, and lower having lowest concentration of anaerobes ($P < 0.001$). A 4 log decrease in total aerobes from upper to lower was unexpected. With approximately 100 cfu/g litter in the lower portion this data implies that the lower of the litter and dirt pad are unexpectedly almost completely devoid of anaerobic bacteria.

^{a,b,c} Numbers with different superscripts are different ($P < 0.0001$).

Error bars represent standard error of the mean, $\pm 0.16 \log_{10}$ cfu/g litter

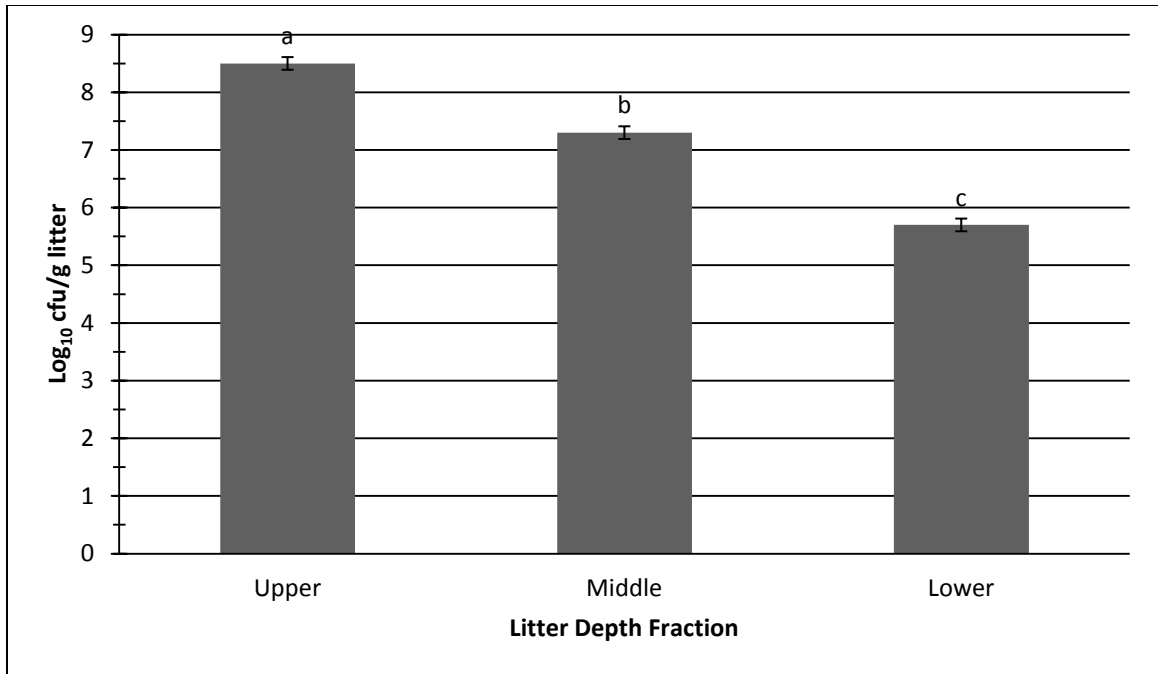


Figure 4. Differences between upper, middle and lower fraction of litter for *Staphylococcus* spp. Results are composite numbers from all sampling locations and times. Differences in *Staphylococcus* spp. concentration were observed between, upper, middle and lower fraction of litter, with upper having higher concentration than either middle or lower, and lower having less than either upper or middle fraction ($P<0.001$). While the upper and middle portions have high quantities of *Staphylococcus* spp. with only a 1 log decrease observed, the lower portion sees 5 and 4 log decreases from the upper and middle respectively. This implies that while broiler litter can harbor substantial concentrations of *Staphylococcus* spp. the lower portion and dirt pad are a very poor environment for this hardy bacterium.

^{a,b,c} Numbers with different superscripts are different ($P<0.0001$).

Error bars represent standard error of the mean, $\pm 0.11 \log_{10}$ cfu/g litter

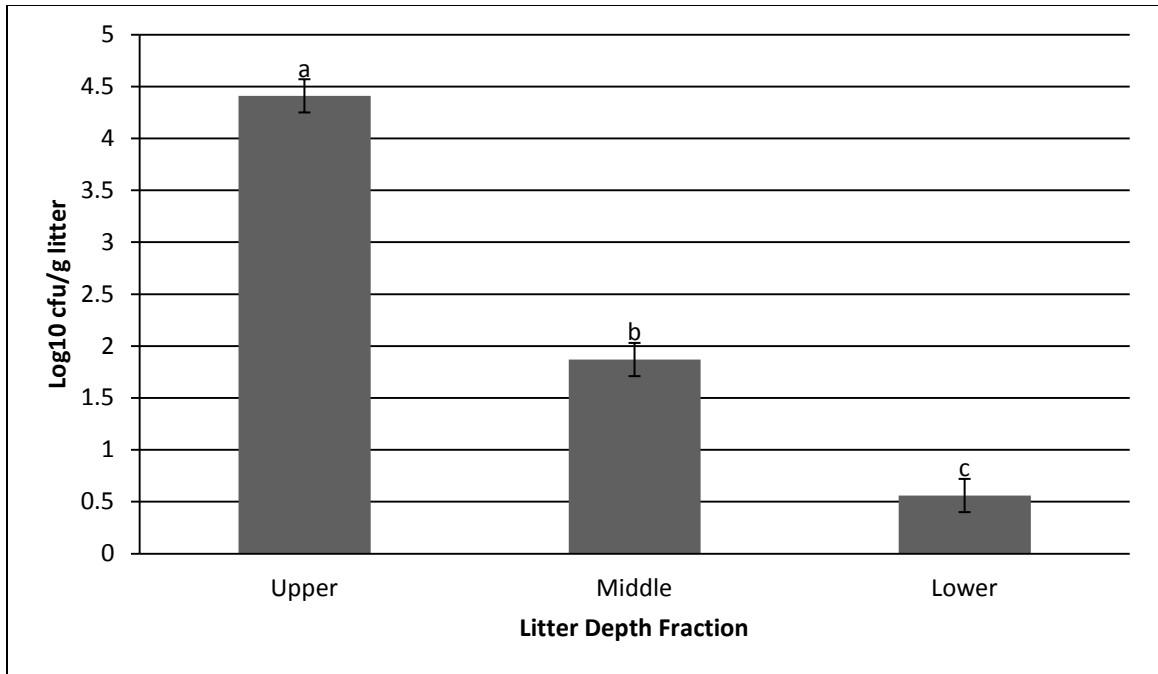


Figure 5. Differences between upper, middle and lower litter fractions for *E. coli*. Results given are composited data from all samples taken. Significant difference was observed between all three fractions, with upper having the highest concentration, then the middle, and lower having the least amount of *E. coli* ($P < 0.001$). *E. coli*, being a common fecal contamination indicator, is almost undetectable in the lower portion of litter and dirt pad because this implies that very little fecal matter reaches the dirt pad.

^{a,b,c} Numbers with different superscripts are different ($P < 0.0001$).

Error bars represent standard error of the mean, $\pm 0.16 \log_{10}$ cfu/g litter

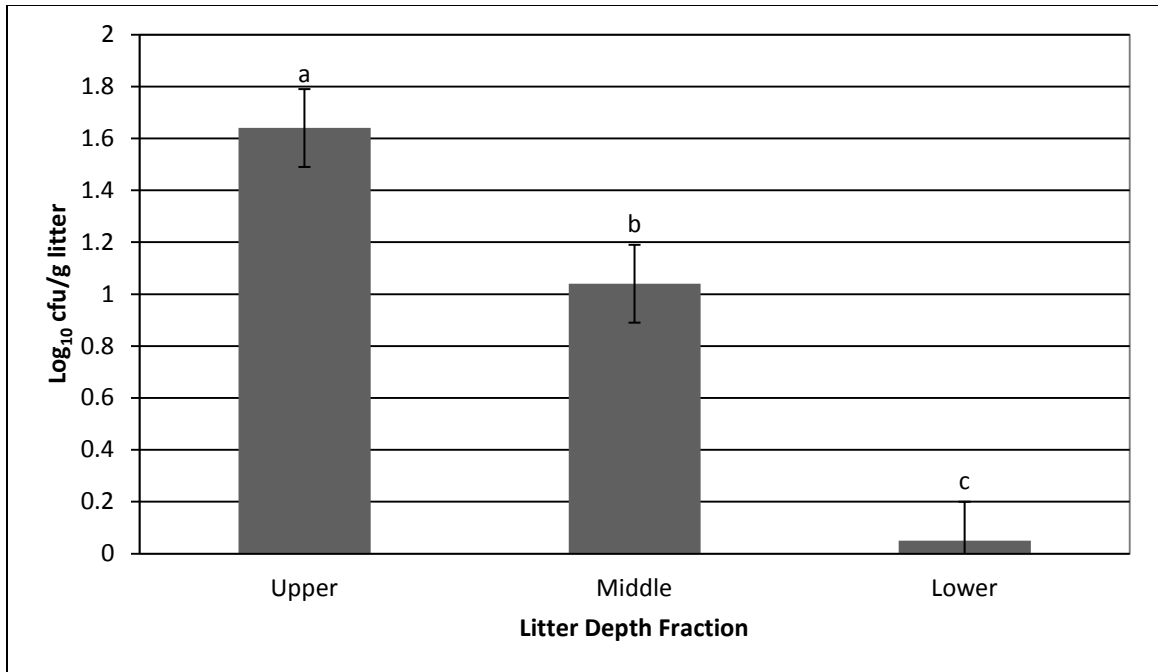


Figure 6. Comparison of *C. perfringens* concentration in upper, middle and lower litter fractions. Data shown is a composite of all samples. Differences were observed between all three fractions, with upper having the highest and lower having the lowest concentration ($P < 0.001$). Despite the lower and dirt pad being thought to have more anaerobic conditions almost no *C. perfringens* was recovered there, and that even spores may not survive in the dirt pad.

^{a,b,c} Numbers with different superscripts are different ($P < 0.0001$).

Error bars represent standard error of the mean, $\pm 0.15 \log_{10}$ cfu/g litter

Table 3. Differences in broiler litter bacteria concentration at three different locations within a commercial broiler grow out house; exhaust fans (EF), middle of house (M), evaporative cooling cells (ECC) (Log₁₀ cfu/g litter)

	Aerobic	Anaerobic	<i>Staphylococcus</i> spp.	<i>E. coli</i>	<i>C. perfringens</i>
EF	7.5 ^{yz}	4.5	7.1	2.5	0.9
M	7.6 ^z	4.4	7.2	2.2	1.0
ECC	7.3 ^z	4.3	7.2	2.0	0.7
SEM	0.08	0.16	0.11	0.15	0.15
<i>P</i> Values	0.0536	0.7649	0.6921	0.0944	0.4802

Total aerobic bacteria were higher in the middle of the house than the evaporative cooling cells ($P=0.0536$). Probable cause of higher bacteria numbers in middle of house due to center house brooding. No other differences between locations of house were observed for remaining bacteria ($P>0.05$).

^{y,z} Numbers with different superscripts are different ($P<0.05$).

SEM = standard error of the mean

The effect of sodium bisulfate on *Salmonella* viability in broiler litter

ABSTRACT

Controlling *Salmonella* populations on commercial broiler grow out farms is a crucial step in reducing *Salmonella* contamination in processing plants. Broiler litter harbors many species of pathogenic bacteria including *Salmonella*. Sodium bisulfate has been shown to reduce concentration of bacteria in broiler litter. In experiments 1 and 2 sodium bisulfate was applied to broiler litter at rates that are comparable to what is commonly used by the poultry industry; 22.7, 45.4, 68.0 kg /92.9 m², after application sodium bisulfate was mixed into litter. In experiments 3 and 4 sodium bisulfate was applied at 45.4 kg/92.9 m², to the surface of the litter. For all experiments a cocktail of 5 *Salmonella* serovars was applied to the litter. Ammonia, pH, moisture, and water activity measurements were taken; additionally, total aerobic, anaerobic, enteric, and *Salmonella* concentrations were determined at 0, 24 and 96 hours. In experiments 1 and 2 *Salmonella* concentrations were higher for treated litter than the control, at 24 and 96 hours ($P<0.001$). In experiments 1 and 2 litter pH was lower for treated litter at 24 and 96 hours, lowest pH was observed with the 68.0 kg/ 92.9 m², with a pH of 5.95 ($P<0.001$). In experiments 3 and 4 litter pH was lowered for treated litter to 2.1 ($P<0.001$). Even this lower pH did not reduce *Salmonella* concentrations compared to the control ($P=0.05$). The decreased litter pH appeared to be responsible for increased viability of *Salmonella*. This research shows that the lowering of litter pH which

decreases litter ammonia production could actually lead to an increased survivability of certain bacteria, like *Salmonella*.

Introduction

Salmonella spp. account for an estimated one million cases of food borne illnesses in the United States per year, with approximately 95% being food borne in origin (Scallan *et al.*, 2011). *Salmonella* recovery in broiler litter is sporadic, since it is not normally in the litter. Lu *et al.* (2003) detected no *Salmonella* by culture methods and PCR in broiler litter. In contrast, *Salmonella* was detected in 83% of farms that reuse litter and 68% of farms that dispose of litter after a single flock of broilers on Australian broiler farms (Chinivasagam *et al.*, 2010). The concentrations of *Salmonella* in each litter sampled varied, with almost a 5 log difference between some litter sampled, the researchers attributed this to several factors including transmission from parent flocks and environmental conditions (Chinivasagam *et al.*, 2010). Comparison of soil characteristics, such as pH, and their effects on *Salmonella* incidence in broiler litter yielded 29% of litter samples being positive, with higher *Salmonella* concentrations observed with lower soil pH (Volkova *et al.*, 2009).

In a survey of bacteria in poultry litter, it was found that samples with lower pH had lower bacterial counts (Terzich *et al.*, 2000). The use of litter amendments to reduce ammonia emissions is a common practice in broiler houses (Terzich *et al.*, 1998). These amendments reduce ammonia by direct chemical interactions, reduction in litter pH and reduced numbers of ammonia producing bacteria (Terzich *et al.*, 1998).

While litter amendments effects on overall bacterial numbers have been shown, its use against *Salmonella* has not been fully characterized. Payne *et al.* (2007) found greatest reduction in *Salmonella* populations with litter pH of 4 or less, and no reduction at pH of 7 and 9. When birds were raised on litter treated with varying levels of aluminum sulfate (3.63 or 7.26 kg/ 4.6 m²) or sodium bisulfate (1.13 or 1.81 kg/4.6 m²), which lowered litter pH to less than 5, there was no reduction in *Salmonella* concentrations or frequency for these birds' whole carcass rinses or ceca samples (Line 2002). Commercially available sodium bisulfate applied at the manufacturer's guidelines (2.27 kg /9.29 m²) also yielded no effect on *Salmonella* populations in broiler litter, even though a pH of 1.2 was achieved in the treated litter (Pope and Cherry, 2000). Payne *et al.* (2002) used granulated sulfuric acid and sodium bisulfate at various application levels on litter that had been placed in baking pans and inoculated with *Salmonella*. High levels of both granulated sulfuric acid and sodium bisulfate (45.3 kg/ 92.9 m²) were needed to obtain litter pH low enough to reduce *Salmonella* concentration (Payne *et al.*, 2002).

Salmonella has various mechanisms by which it can survive acidic environments, such as the stomach (Perez and Groisman, 2007; Lee *et al.*, 1994; Foster and Hall, 1990). Acid tolerance of *Salmonella* has been linked to synthesis of several proteins that protect cells in acidic environments. These mechanisms when activated in such environments allow *Salmonella* to survive in pH as low as 3.3 (Foster and Hall, 1990, Lee *et al.*, 1994). Virulence of *Salmonella* has been linked to acid resistance (Foster and Hall, 1990). The researchers found that virulent strains of *Salmonella* were more resistant to acidic environments than avirulent strains.

The goal of this research was to determine any differences for varying application rates and application methods of sodium bisulfate to broiler litter, and observe any differences in *Salmonella* concentrations.

Materials and Methods

Experiments 1 and 2 were performed in May and June of 2010. Experiments 3 and 4 were performed in February and April of 2011. All experiments were identical, except that a different litter source was used in each trial. Each experiment was carried out for 96 hours from initial addition of *Salmonella spp.*, and treatment with sodium bisulfate.

Five *Salmonella* species used in these experiments were *Salmonella* Enteritidis, *Salmonella* Montevideo, *Salmonella* Heidelberg, *Salmonella* Typhimurium, and *Salmonella* Kentucky. These *Salmonella* were isolated from either commercial broiler farms, or processing plants. *Salmonella* were isolated from carcass rinses, processing plant, or cloacal swabs, farm, enriched in tetrathionate broth, at 37° C for 48 hours. After enrichment XLT4 plates were streaked for isolation, using standard protocols, then incubated at 37°C for 24 hours. Suspect *Salmonella* were identified using 16S rRNA sequencing. Cultures were stored frozen at -80° C until needed. When needed frozen cultures were streaked onto Tryptic Soy Agar containing 5% sheep red blood cells and incubated at 37° C for 24 hours. A single colony was picked at 24 hours and streaked onto Brain Heart Infusion Agar Slants, then incubated at 37° C for 24 hours. Slants were kept for future use. Brain Heart Infusion Broth cultures were made from streaking a colony from the Brain Heart Infusion Agar slants and incubating them at 37° C for 24

hours with constant shaking. For each of the five serovars, 6 ml of Brain Heart Infusion Broth was taken and combined into a 30 ml cocktail that would be applied to litter.

Sufficient quantity of pine shaving broiler litter, that had been used for at least 2 grow out periods on the Auburn Poultry Research Farm, was obtained weighed and stored in a heated room (27° C). Litter moisture was determined at least five days before initiation of each experiment, by taking three samples from the litter, and drying as described below. From the percent moisture obtained it was possible to determine the amount of water to add to equilibrate the litter moisture to 25-30%. This water was added 72 hours before initiation of the experiment. As water was added the pile was turned to allow even dispersal of water. The litter was kept in a heated (27° C) room for the duration of the experiment to facilitate ammonia production.

Initially three kg of litter was placed into one of twelve 0.53 x 0.39 m (0.16 m²) Rubbermaid plastic tubs. Litter was allowed to equilibrate for 1 hour before any measurements were taken or treatments added. After equilibration initial samples were taken, the *Salmonella* cocktail and then sodium bisulfate were applied to the litter. Each litter replication received 30 ml of *Salmonella* cocktail, at 10⁹ cfu/ml.

For experiments 1 and 2 sodium bisulfate was applied to the litter in three replicate tubs at a rate of either 22.7, 45.4, or 68.0 kg /92.9 m². Each application rate was applied to three boxes, with 3 untreated replications serving as controls. Sodium bisulfate was applied to the litter by evenly distributing it on top of the litter and mixing it into the litter by hand, *Salmonella* cultures were added similarly. Grab samples were taken in which approximately 50 g of litter was collected by hand from each of the four

corners and the center of the plastic tub. For each tub the five samples were thoroughly mixed before analysis. After sample removal, the remaining litter was evenly spread in the container to maintain a flat surface area.

Sodium bisulfate in experiments 3 and 4 was applied at a rate of 45.4 kg/92.9 m² for all treatments. For treatment 1, litter was allowed to equilibrate for 1 hour in a plastic tub, and then the *Salmonella* cocktail and sodium bisulfate were added and mixed into the litter as described above. For treatment 2 and 3 litter was placed into tubs, *Salmonella* was added and then allowed to sit for 24 hours, after 24 hours sodium bisulfate was applied to the litter surface, and not mixed into the litter. For the control, litter was allowed to equilibrate for 1 hour, after which the *Salmonella* cocktail was added. Samples from treatment 1, 3 and the control were taken by the grab method described for trials 1 and 2. Samples from treatment 2 were taken by scraping the heel of the hand across the surface of the litter. Treatments and sampling methodology are given in Table 4.

Litter pH was obtained by taking 5 g of litter to which 45 ml of distilled water was added after allowing 1 hour to equilibrate, pH readings were taken with a Fischer Scientific Accumet pH meter 50 (Denver Instrument Company, Bohemia, NY, USA). Litter moisture was obtained by taking 10 g of litter, and drying for 48 hours at 100 C. Using the weight loss between initial and dried litter the percent moisture was calculated.

Ammonia measurements were taken at 0, 24, 48, 72 and 96 hours post treatment. Dräger Chip Measuring System was used, with a plastic rectangular tub (17 x 25 x 12 mm) attached to pump (Dräger Safety, Inc., Lubeck, Germany). Tub was placed directly

onto the litter and pump was allowed to run for 60 seconds before a measurement was taken. After each measurement the collecting tub was purged with fresh air by moving the tub with pump on up and down 4 to 5 times.

For bacterial recovery, 10 g of litter from 0, 24 and 96 hour samples was obtained. This litter was placed in sterile filter bags with 90 ml of Phosphate Buffered Saline, and stomached for 60 seconds. Additional serial dilutions were made by taking 1 ml and adding it to 9 ml of Phosphate Buffered Saline, after which the dilution was vortexed for 5 seconds. This was carried out for sequential dilutions, until the appropriate dilutions had been achieved. For each dilution, 0.1 ml was spread plated, in duplicate, onto appropriate media types for each specific bacterium. These plates were inverted and incubated at 37° C for 24 hours; aerobic bacteria, *Salmonella* and *E. coli* were incubated in a normal atmosphere while anaerobic bacteria were incubated in an atmosphere consisting of 5% CO₂, 5% H₂, and the balance in N₂. Since bacterial recovery methods were not performed strictly anaerobically, any anaerobic bacteria recovered will be classified as either facultative anaerobes or anaerobic spore formers.

Media utilized were; Plate Count Agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) for total aerobic bacteria; Anaerobic Agar (Difco™) for total anaerobic bacteria; MacConkey's Agar (Difco™) for *E. coli*. *Salmonella* recovery was performed by two methods, direct plating and enrichment. Direct plating was performed as described onto Xylose-Lysine-Tergitol 4 (XLT4 agar, Difco™). Enrichments were performed in Tetrathionate Broth (Difco™) for 48 hours, and then streaked onto XLT4 to ensure that any sample with *Salmonella* concentrations below enumeration limits were not falsely identified as negative.

Data were analyzed using SAS 9.2. using the general linear model at the 0.05 level of significance (SAS Institute 2009). Litter moisture percentages were arcsine transformed, and cfu/g litter transformed to Log₁₀. Means were separated using Tukey's Honestly Significant Difference test. Data were analyzed for effects due to, sodium bisulfate level or application method, sampling time, trial, and interaction between sodium bisulfate level or application level and sampling time. If no difference was found due to main effects or interactions between experiments data was combined.

Results

Experiments 1 and 2

Total aerobic counts were not affected by level of sodium bisulfate added to the litter ($P>0.05$). There was an increase in total aerobes between 24 and 96 hours, 8.57 and 9.44 log₁₀ cfu/g litter respectively ($P<0.001$). Total facultative anaerobes were reduced by the 22.7 kg/ 92.9 m² treatment ($P=0.037$).

E. coli was affected by sodium bisulfate level ($P= 0.02$) and trial ($P<0.001$). Sodium bisulfate applied at 45.4 kg / 92.9 m² had higher levels of *E. coli* than the control, 6.4 and 4.8 log₁₀ cfu / g of litter respectively. *E. coli* levels were only affected by trial ($P<0.001$), where in the second trial there was no recoverable *E. coli* (Data not shown).

Salmonella concentrations before the addition of the *Salmonella* cocktail (0 hour sampling) were below detectable limits, by both plating and enrichment. At 24 hours post initial treatment, *Salmonella* concentration for control was 3.0 log₁₀ cfu/g litter, while concentrations for 22.7, 45.4, 68.0 kg /92.9 m² application rates was 5.7, 5.9, and 5.0 log₁₀ cfu/g litter, respectively. At 96 hours control *Salmonella* concentrations were

0.83 log₁₀ cfu/g litter, while concentrations for 22.7, 45.4, 68.0 kg /92.9 M² were 3.57, 4.41, 4.35 log₁₀ cfu/g litter.

An interaction for *Salmonella* was observed between treatment level and sampling time, as shown in Figure 1 A ($P \leq 0.001$). At 24 hours post sodium bisulfate treatment, and *Salmonella* addition *Salmonella* concentrations were higher in sodium bisulfate treated litter, regardless of application rate, than in control litter by at least 2 logs. At 96 hours *Salmonella* in control, 22.7, and 45.4 kg /92.9 M² treatments had decreased from 24 hours. The highest application rate (68.0 kg) had decreased *Salmonella* concentrations but not statistically lower than other treated groups. In the untreated control *Salmonella* levels had been reduced to less than 1 log. This implies that the sodium bisulfate at any level of application can prolong *Salmonella* survival and viability in litter.

Litter pH was affected by the interaction of sodium bisulfate level and time, as shown in Figure 7 B ($P < 0.001$). No decrease was found in the control litter. For the 22.7 and 45.5 kg application rates, pH were less than control and higher than the 68.0 kg rate, for all time periods. These two treatments were never different from each other. A decrease was observed for 22.7, 45.4, 68.0 kg /92.9 m² application rates from 0 hours to 24 hours, and a numerical increase was observed from 24 to 96 hours.

At 0 hours no differences were observed between sodium bisulfate application rates for ammonia levels ($P = 0.968$). At 24, 48, 72 and 96 hours control litter had higher ammonia levels than the treated litter ($P < 0.001$). At 24 hours no differences were observed for treated litter. At 48 hours the 68 kg/92.9 m² application was lower than the 22.7 kg/92.9 m² rate, 22.4 and 67.5 ppm respectively ($P < 0.001$). At 72 hours both the

45.4 and 68 kg/92.9 m² were lower than the 22.7 kg/92.9 m² rate, 52.9, 27.0 and 85.8 respectively ($P<0.001$). At 96 hours ammonia level for the 68 kg/92.9 m² application rate was lower than the 22.7 kg/92.9 m² rate, 37.0 and 86.0 ($P<0.001$). Data shown in Table 5.

Experiments 3 and 4

In experiment 3 and 4, ammonia levels were reduced 24 hours after *Salmonella* addition for all treatments; and the control treatments had higher levels of ammonia when compared to treatments 1, 2 and 3 ($P<0.001$). There was no difference in ammonia between any of the sodium bisulfate treatments at each sampling time. (Data not shown)

For both experiments 3 and 4 initially there were no differences between treatments for total aerobes and anaerobes also no *Salmonella* was recovered (aerobes $P=0.80$, anaerobes $P=0.801$).

In experiment 3 at 24 hours post treatment no difference was observed for aerobic bacteria ($P=0.25$), and *Salmonella* ($P=0.088$). A difference was observed for anaerobic bacteria ($P=0.004$) and *E. coli* ($P<0.001$). Anaerobic bacteria were higher for treatments 1 and 4, 7.1 and 6.9 log₁₀ cfu/g litter, than treatment 2, 5.2 log₁₀ cfu/g litter. There was no *E. coli* recovered for treatment 2, while treatments 1,3 and 4 had *E. coli* concentrations of 5.1, 4.2 and 4.9 log₁₀ cfu/g litter respectively.

In experiment 3 at 96 hours, differences were observed for aerobic bacteria ($P=0.03$), anaerobic bacteria ($P=0.029$), *E. coli* ($P=0.002$), and *Salmonella* ($P=0.05$). Total aerobic and anaerobic bacteria were higher in treatment 4 than treatment 2, 9.6 and 7.0 log₁₀ cfu/g litter for aerobes and 6.6 and 4.6 log₁₀ cfu/g litter for anaerobic bacteria.

Again at 96 hours no *E. coli* was recovered from treatment 2, while treatment 3 had 1.2 log₁₀ cfu/g litter, treatment 1 had 3.3 log₁₀ cfu/g litter and treatment 4 was the highest with 4.9 log₁₀ cfu/g litter. *Salmonella* concentrations in treatment 1 were higher than treatment 2, 4.5 and 0.6 log₁₀ cfu/g litter respectively.

In experiment 4 at 24 hours post treatment there was differences due to treatment for aerobic bacteria ($P=0.018$) and *Salmonella* ($P=0.027$), but no difference was observed for anaerobic bacteria ($P=0.614$). Treatment 4 had a higher concentration of total aerobic bacteria than treatment 2, 8.9 and 8.4 log₁₀ cfu/g litter respectively. *Salmonella* concentration was higher in treatment 3 than treatment 1, 3.7 and 1.3 log₁₀ cfu/g litter respectively.

At 96 hours there was a difference observed for aerobic bacteria ($P=0.009$), anaerobic bacteria ($P=0.031$), and *Salmonella* ($P=0.015$). Total aerobic bacteria were highest for treatment 1, 9.2 log₁₀ cfu/g litter. There was no difference observed between treatments 2, 3 and 4 with 8.5, 8.7 and 8.4 log₁₀ cfu/g litter respectively. Total anaerobic bacteria were higher in treatment 2 than treatment 4, 6.0 and 5.1 log₁₀ cfu/g litter. *Salmonella* were higher in treatment 1 than treatment 2 and 4, 4.0 and 2.4 and 2.0 log₁₀ cfu/g litter. *Salmonella* data for experiments 3 and 4 given in table 6A.

There was 2 factor interaction between treatment and time for experiments 3 and 4 with regards to litter pH ($P<0.001$). In both experiments litter pH was reduced from 0 to 24 hours in litter that had been treated with sodium bisulfate. Regardless of sampling time litter pH in sodium bisulfate treated litter was less than control litter. In experiment 3, treatment 2 and 3 had lower litter pH than treatment 1. In experiment 4 treatment 2

had lower litter pH than both treatments 1 and 3, which were not different from each other. At 96 hours litter pH in experiment 3 was lower in treatment 2 and 3 than treatment 1. In experiment 3 litter pH was lower in treatment 2 than treatment 1. Litter pH for experiments 3 and 4 shown in table 6B.

No differences were observed between treatments for moisture content for any experiment (Experiment 1 and 2 $P=0.5288$, Experiment 3 and 4 Data not shown).

Discussion

As observed in experiments 1 and 2, as litter pH is decreased due to the application of sodium bisulfate, the survivability of *Salmonella* increased. Past studies have shown application of sodium bisulfate to decrease the pH of litter to much lower levels than found in this study (Payne *et al.*, 2002, Payne *et al.*, 2007). Payne *et al.* 2002 found that after 24 hours at the application rate of 45.4 kg/92.9 m², litter pH was reduced to 3.4. This application rate was also found to reduce *Salmonella* concentrations; this reduction in pH and *Salmonella* is in disagreement with the findings presented here. The 45.4 kg/92.9 m² application rate for experiments 1 and 2 did not reduce litter pH as much, only to 7.03 at 24 hours. A pH of 7.03 is a physiologically neutral pH, and would benefit *Salmonella*. Payne *et al.* (2002) attributed the observed decrease in *Salmonella* to acidified litter, with higher sodium bisulfate application rates. In agreement with Payne *et al.* (2002), this study found that when litter pH is reduced to a neutral pH that *Salmonella* viability is not reduced, but may actually be increased. Line (2002), found no difference in *Salmonella* colonization in broilers reared on litter treated with sodium bisulfate at the application rates of 22.9 and 36.2 kg/ 92.9 m². These researchers also

found a reduction in litter pH but no reduction in *Salmonella* colonization. Why the litter pH observed here was so much higher than other reported litter pH with the same or very similar sodium bisulfate application rates is due to application and sampling method. Sodium bisulfate was mixed into the litter and grab samples were taken. In previous studies, sodium bisulfate was surface applied and surface samples were taken (Payne *et al.*, 2002).

In experiments 3 and 4 application method of sodium bisulfate, either surface applied or mixed into litter, decreased ammonia levels after initial treatment but surprisingly there was no difference between the two methods. It was expected that surface applied sodium bisulfate would reduce ammonia emissions more effectively. Application method did effect *Salmonella* concentrations at 24 and 96 hours after treatment. In experiment 4, at 24 hours treatment 1 had lower concentrations of *Salmonella* compared to treatment 3. In experiments 3 and 4 the situation is reversed at 96 hours, with treatment 1 having higher concentrations of *Salmonella*. In experiment 3 the difference in *Salmonella* levels between treatment 1 and treatment 2 can be attributed to sampling method, as there is no difference in *Salmonella* between surface applied sodium bisulfate with grab sampling and mixed in sodium bisulfate or surface application with heel scrape sampling technique. This demonstrates a potential bias due to sampling technique. In experiment 4 despite differences in pH at 24 hours between treatments there is no difference for *Salmonella*. An increase in *Salmonella* was observed between 24 hours and 96 hours when sodium bisulfate was mixed into the litter, however no treatment had lower *Salmonella* levels than the control.

Salmonella's acidic pH survival mechanisms have been observed to allow it to survive in acidic environments as low as pH 3.3 (Foster and Hall 1990; Lee *et al.*, 1994). *Salmonella* that has been subjected to acidic pH levels have been observed to exhibit increased virulence (Foster and Hall 1990) and increased antibiotic resistance (Perez and Groisman 2007). The pH values observed in experiments 1 and 2 did not reach acidic levels, but remained at a neutral level; the highest application rate of 68.0 kg /92.9 M² had the lowest pH at 5.9, a slightly acidic but still neutral pH. In experiments 3 and 4 litter pH reached an acidic level, lowest 2.11, but even this pH still did not reduce *Salmonella* concentration. The genetic defense mechanisms of *Salmonella* should have been able to resist this pH change and allow the bacterium to survive in litter as observed. Data presented here shows that in the complex environment of broiler litter, a combination of treatments or approaches are needed to reduce ammonia as well as potentially pathogenic bacteria.

Commercially available sodium bisulfate is commonly used in broiler grow out houses to reduce ammonia volatilization from litter; the data in this paper shows that in this regard the chemical works very well. What the data presented in this paper disputes is the ability of sodium bisulfate to reduce *Salmonella* in broiler litter. Previous research has indicated application of sodium bisulfate to substantially reduce or eliminate *Salmonella*, however the observations made here showed no reduction in *Salmonella* regardless of application method or application rate. This research suggests the need for further research into efficacy of sodium bisulfate to control *Salmonella* in broiler litter.

References

- Chinivasagam, H. N., M. Redding, G. Runger and P.J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. *Br. Poult. Sci.* 51:311-318.
- Foster, J. W. and H. K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* 172: 771-778.
- Lee, I. S., J. L. Slonczewski, and J. W. Foster. 1994. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. *J. Bacteriol.* 176:1422-1426.
- Line, J. E. 2002. *Campylobacter* and *Salmonella* populations associated with chickens raised on acidified litter. *Poult. Sci.* 81:1473-1477.
- Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16s rRNA and functional gene markers. *Appl. Environ. Microbiol.* 69(2):901-908.
- Payne, J. B., E. C. Kroger and S. E. Watkins. 2002. Evaluation of litter treatments on *Salmonella* Recovery from Poultry litter. *J. Appl. Poult. Res.* 11:239-243.
- Payne, J. B., J. A. Osborne, P. K. Jenkins and B. W. Sheldon. 2007. Modeling the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and water activity. *Poult. Sci.* 86:191-201.
- Perez, J. C. and E. A. Groisman. 2007. Acid pH activation of the PmrA/PmrB two-component regulatory system of *Salmonella enterica*. *Mol. Microbiol.* 63:283-293.
- Pope, M. J. and T. E. Cherry. 2000. An evaluation of the presence of pathogen on broilers raised on Poultry Litter Treatment®-treated litter. *Poult. Sci.* 79:1351-1355.
- SAS Institute. 2009. SAS User's Guide. Statistics. Version 9.1 ed. SAS Inst. Inc., Cary, NC.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7-15.
- Terzich, M., C. Quarles, M. A. Goodwin and J. Brown. 1998. Effect of poultry Litter Treatment® (SODIUM BISULFATE®) on death to ascites in broilers. *Avian Dis.* 42:385-387.

Terzich, M., M. J. Pope, T. E. Cherry and J. Hollinger. 2000. Survey of pathogens in poultry litter in the United States. *J. Appl. Poult. Res.* 9:287-291.

Volkova, V. V., R. H. Bailey and R. W. Willis. 2009. *Salmonella* in broiler litter and properties of soil at farm location. *PLoS ONE.* 4:6403.

Table 4. Description of treatments used in experiments 3 and 4

	Treatment			
	1	2	3	4
Sodium bisulfate Application Method	Thoroughly mixed into litter by hand	Surface Applied	Surface Applied	None
Hours Between <i>Salmonella</i> Application and Sodium bisulfate Application	0	24	24	0
Sampling Technique	Grab sampling from the 4 corners and center of box	Surface scrape with heel of hand	Grab sampling from the 4 corners and center of box	Grab Sampling

Treatment numbers are given on the horizontal axis; 1, 2, 3 and 4, with application method, hours between *Salmonella* and sodium bisulfate application, and sampling technique on the vertical axis. These treatment numbers will be used for experiments 3 and 4.

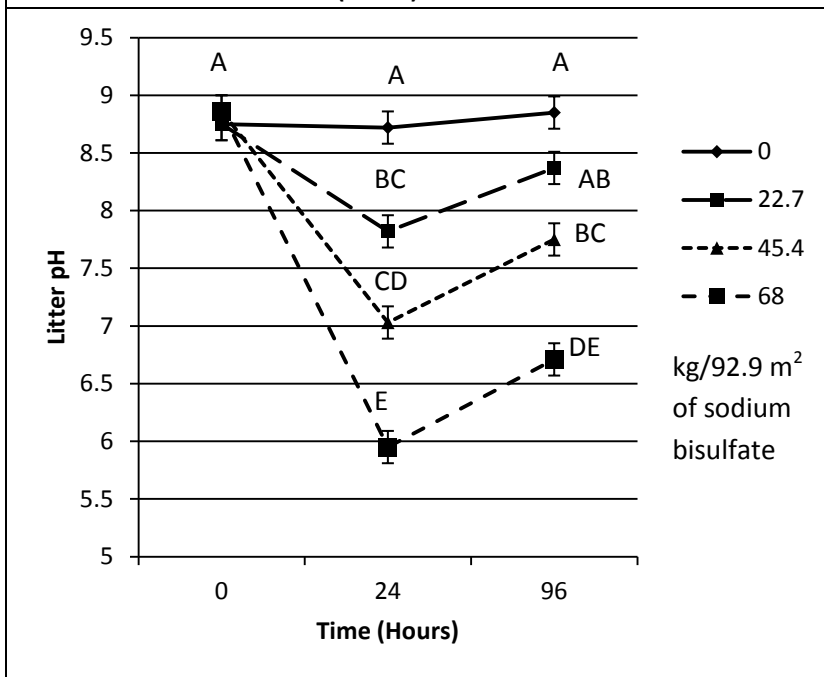
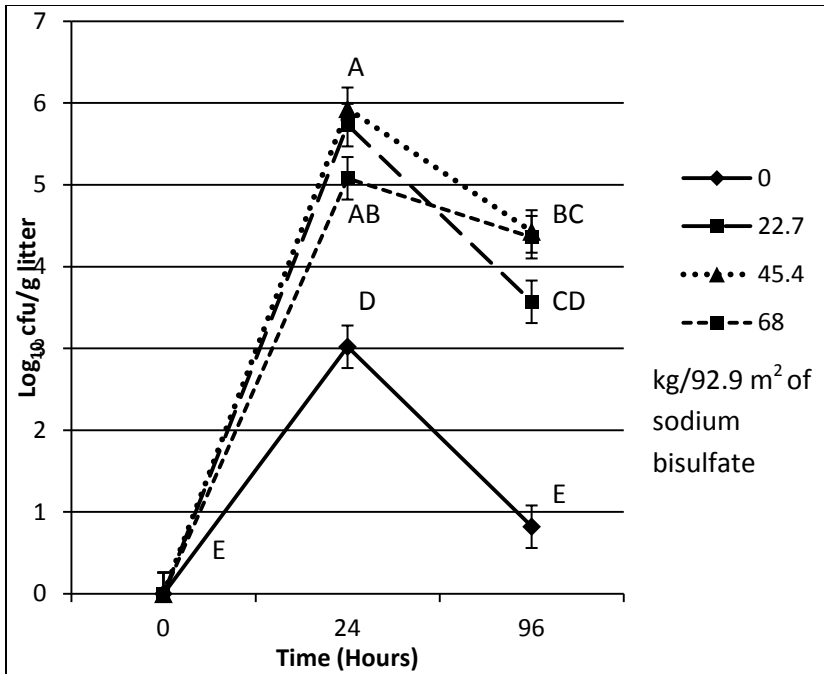


Figure 7. *Salmonella* concentrations and litter pH for experiments 1 and 2. Time post treatment given in hours on the horizontal axis, and \log_{10} cfu/g litter (Figure 1A) or litter pH (Figure 1B) given on the vertical axis.

A) *Salmonella* concentration was affected by 2 way interaction between sampling time and sodium bisulfate application rate ($P < 0.001$). Populations decreased to barely detectable levels, while *Salmonella* concentrations in sodium bisulfate treated litter remained high for the duration of experiment. B) Litter pH was effected by 2 way interaction between sampling time and sodium bisulfate supplication rate ($p < 0.001$). Increased amount of sodium bisulfate applied led to decrease in litter pH at both sampling times. Litter pH decreased from 0 to 24 hours, and increased from 24 to 96 hours.

^{a-e}Numbers with different superscripts are different ($P < 0.05$).

Table 5. Average ammonia levels (ppm) for each treatment at 0, 24, 48, 72 and 96 hours for experiments 1 and 2.

Sodium Bisulfate kg/92.9 m ²	Time (Hours) Post Treatment				
	0	24	48	72	96
0 (Control)	426.6	199.8 ^a	207.0 ^a	158.1 ^a	136.5 ^a
22.7	411.3	41.6 ^b	67.5 ^b	85.8 ^b	86.0 ^b
45.4	422.3	27.0 ^b	47.7 ^{bc}	52.9 ^c	54.97 ^{bc}
68	436.6	16.6 ^b	22.4 ^c	27.9 ^c	37.0 ^c
SEM	36.6	7.6	8.0	7.4	8.1
P Value	0.968	<0.001	<0.001	<0.001	<0.001

No difference for ammonia was observed at 0 hours ($P=0.968$). At 24, 48, 72 and 96 hours post treatment there was a difference between all application rates and control litter ($P<0.001$). At 48 and 96 hours 68 kg/92.9 m² application rate had lower ammonia than 22.7 kg/92.9 m² application rate ($P<0.001$). At 72 hours the 45.4 and 68 kg/92.9 m² application rates were lower than the 22.7 kg/92.9 m² rate, but were not different from each other ($P<0.001$).

^{a-d}Numbers with different superscripts are different ($P < 0.05$).

Table 6. Difference in *Salmonella* concentrations and litter pH for experiments 3 and 4.

A.

Treatment	Experiment					
	3			4		
	0	24	96	0	24	96
1	0	4.0	4.5 ^a	0	1.3 ^b	4.0 ^a
2	0	1.1	.7 ^b	0	2.5 ^{ab}	2.4 ^b
3	0	2.7	2.0 ^{ab}	0	3.8 ^a	2.5 ^b
4	0	3.7	2.9 ^{ab}	0	3.2 ^{ab}	2.0 ^b
SEM	0	0.42	0.47	0	0.26	0.12
<i>P</i> value	1	0.09	0.05	1	0.027	0.015

B.

Treatment	Experiment					
	3			4		
	0	24	96	0	24	96
1	8.51 ^a	5.98 ^b	5.61 ^b	8.98 ^a	6.81 ^{bc}	7.51 ^{ab}
2	8.51 ^a	2.69 ^{cd}	2.11 ^d	8.98 ^a	3.70 ^d	5.26 ^{cd}
3	8.51 ^a	3.40 ^c	2.62 ^{cd}	8.98 ^a	5.90 ^{bc}	6.51 ^{bc}
4	8.51 ^a	7.85 ^a	7.94 ^a	8.98 ^a	8.85 ^a	8.78 ^a
SEM		0.117			0.181	
<i>P</i> value		<0.001			<0.001	

Table 6. A) Differences between *Salmonella* concentrations (\log_{10} cfu/g litter) at each sampling time for experiments 3 and 4. In experiment 3 no difference at 24 hours, however at 96 hours there is a difference between treatments 1 and 2. ($P = 0.05$). In experiment 4 treatment 1 had lower *Salmonella* concentration than treatment 3, at 24 hours ($P = 0.027$). At 96 hours, treatment 1 had higher *Salmonella* concentration than any other treatment ($P = 0.015$) B) Two factor interaction between time and treatment for pH for experiments 3 and 4. A decrease was observed between 0 and 24 hours for

treatments 1, 2 and 3. Treatment 1 pH was higher than 2 and 3 in experiment 3 and higher than treatment 2 in experiment 4 at 24 and 96 hours.

^{a-d}Numbers with different superscripts at each time are different ($P < 0.05$).

Reduction of *Salmonella* and ammonia emissions in broiler litter using sulfuric acid and aluminum sulfate.

Abstract

In recent years an emphasis has been placed on reducing foodborne pathogens on the farm. Of particular interest to the poultry industry is *Salmonella enterica*, one potential reservoir for this bacterium on the farm is in the litter. In the United States, a common practice in broiler management is the reuse of litter for several consecutive flocks. If one flock is colonized with *Salmonella* and the bacterium is then subsequently shed into the litter, *Salmonella* free flocks will be colonized once placed onto that litter. Another problem associated with intensively reared broiler chickens is the high level of ammonia volatilized from litter during the life of the flock. High ammonia emissions can negatively impact broiler health, performance and even the health of farm workers.

Numerous litter amendments have been developed that reduce ammonia emissions and potentially *Salmonella*. In this section, two experiments are described in which built up litter was added to a box and then a chemical treatment was added. In the first experiment, sulfuric acid was chosen to determine its potential for reducing *Salmonella* and ammonia. Sulfuric acid was applied to litter at three application rates of 9.07, 18.14 and 27.21 L/92.9 m². In experiment two, commercially available aluminum sulfate was applied to litter at three different rates; 22.7, 45.5 and 68.0 kg/92.9 m². In addition to the treatments, a cocktail of five poultry associated *S. enterica* serovars was

also applied to the litter. *Salmonella*, ammonia, moisture, pH and water activity (A_w) levels were monitored immediately before treatment and every 24 hours for 96 hours.

For sulfuric acid treated litter, a reduction was seen in ammonia levels 24 hours after application regardless of application rate from an initial level greater than 200 ppm to less than 4 ppm, this reduction continued for the duration of the experiment ($P < 0.001$). After 24 and 96 hours, no *Salmonella* was detected from any of the treated litter. In litter treated with aluminum sulfate, there was a significant reduction in ammonia starting at 24 hours after treatment and continuing for the duration of the experiment; with no observable differences between application rates ($P < 0.001$). *Salmonella* was recovered in all litter replicates that were treated with aluminum sulfate, with no difference observed in cfu's between treated and untreated litter. The data presented here indicates that sulfuric acid and aluminum sulfate are suitable candidates for reducing ammonia emissions, and indicates sulfuric acid as a candidate for *Salmonella* reduction.

Introduction

Poultry meat is the leading source of food borne illness in the United States accounting for 1.5 million total cases, nearly 12,000 hospitalizations and 180 deaths per year (Batz *et al.*, 2011). Of these cases, it is estimated that 35.1% are caused by *Salmonella spp.* Recently it has been shown that *Salmonella* has been recovered from 83% of Australian broiler chicken farms that reuse litter (Chinivasagam *et al.*, 2010). In a study of the effects of soil characteristics on incidence of *Salmonella* in litter, nearly a third (29%) of litter samples tested were *Salmonella* positive (Volkova *et al.*, 2009).

One of the problems that has arisen with the intensive rearing conditions associated with broiler chicken production is the high level of ammonia that can be volatilized from litter. This ammonia is produced by the ureolytic action of bacteria found in the litter (Rothrock *et al.*, 2010). Eight hour exposure to ammonia levels higher than 25 ppm has been banned by the United States Occupational Safety and Health Association. Ammonia in high enough concentrations (100 ppm) or greater can cause lesions to the mucosal linings of the nasal passages, esophagus and lungs, which will in turn lead to a significant reduction in bird live performance (Fulton, 2008). Ammonia emissions from litter can be reduced to acceptable levels by the application of acidifying litter amendments, more specifically acidifiers. Acidifying litter amendments work by reducing litter pH and trapping ammonia as ammonium, these amendments can also reduce the number of ureolytic bacteria present (Terzich *et al.*, 1998).

It has also been shown that acidifying litter amendments reduce *Salmonella* concentrations in litter if a low enough pH can be achieved. Some of these amendments have shown reduction of *Salmonella* at a litter pH of 4 or less (Payne *et al.*, 2007; Pope and Cherry 2000; and Payne *et al.*, 2002). These researchers applied either aluminum sulfate, sodium bisulfate or granulated sulfuric acid to achieve the low pH's needed to first trap ammonia and secondly reduce *Salmonella*. These chemicals work by trapping ammonia as an ammonium salt. At higher litter pH levels, 5 or higher, it has been observed that no reduction in *Salmonella* was observed (Williams *et al.*, 2012; Payne *et al.*, 2007; Line, 2002).

Salmonella in contaminated food must first pass through the acidic environment of the stomach before it can enter the small intestine and cause illness. The production of

acid shock proteins in response to acidic environments enables *Salmonella* to adapt and survive (Foster, 1993). It has also been found that when *Salmonella* is adapted to an acidic environment its virulence can increase, researchers noted that virulent strains can survive better in acidic environments such as the stomach (Foster and Hall, 1990).

The goal of these experiments was to determine the effectiveness of sulfuric acid and aluminum sulfate at reducing ammonia emissions and *Salmonella* concentrations in broiler litter. It was hypothesized that due to the acidic nature of both treatments, they would be highly effective at reducing litter pH, trapping ammonia and eliminating or reducing *Salmonella* concentrations.

Materials and Methods

Five *Salmonella* species were used in this experiment; *Salmonella* Enteritidis, *Salmonella* Montevideo, *Salmonella* Heidelberg, *Salmonella* Typhimurium, and *Salmonella* Kentucky. The *Salmonella* isolates were previously cultured from either commercial broiler houses or processing plants and identified by 16S rRNA sequencing. Cultures were stored in glycerol solution at -80° C, when needed cultures were streaked onto Tryptic Soy Agar containing 5% sheep red blood cells (BBL™, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and incubated aerobically at 37° C for 24 hours. After incubation, a single isolated colony was picked and streaked onto Brain Heart Infusion Agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) slants, and incubated as above. These slants were stored at 4°C for future use. Brain Heart Infusion Broth (Difco™) was inoculated from the above mentioned Brain Heart Infusion slants. This broth culture was incubated at 37°C for 24 hours with

shaking (150 rotations per minute). A cocktail of the five *Salmonella* species was made by combining 6 ml of each species, making a 30 ml cocktail of 10^9 cfu/ml that was applied to the litter. *Salmonella* concentrations were enumerated by spread plating 0.1 ml of each serovar onto Plate Count Agar (Difco™).

Broiler litter, composed of pine shavings and poultry manure, was obtained, weighed and stored in a heated room (27° C) at the Auburn University Poultry Science Research Unit. Five days before the start of the experiment, litter moisture was determined by taking three samples from the litter. Ten grams of litter from each sample was dried at 110° C for 48 hours and then reweighed, to obtain percent moisture. From these results, water was added to adjust litter moisture to 25-30%, which is the level typically observed on a commercial broiler farm (Martin and McCann, 1998). As the water was added, litter was turned to facilitate even dispersal. After addition of the water, litter was heated (27° C) for 72 hours to enhance ammonia production.

On the day of the experiment, three kg of litter was placed into one of twelve identical plastic tubs each measuring 0.53 m x 0.39 m. After placement in plastic tubs, litter was allowed to equilibrate for one hour before any samples were taken or treatments added. Initial samples were taken after equilibration, and the *Salmonella* cocktail was applied to each replicate.

After addition of *Salmonella*, one of four possible treatments was utilized; either water (negative control), sulfuric acid at a rate of 9.07, 18.14, or 27.21 liters (L)/92.9 m². Sulfuric acid was mixed with water to obtain treatments totaling, 75.6, 151.2 or 226.8 L/92.9 m². This was done to prevent a violent reaction when applying to poultry litter.

Commercially available aluminum sulfate (Al⁺Clear[®] Poultry Grade Alum) was applied to litter in one of four possible application rates: 0 (negative control), 22.7, 45.4 or 68 kg/92.9 m². The granulated aluminum sulfate was evenly distributed on the litter surface, and then incorporated into the litter by thoroughly mixing with a gloved hand.

Litter samples were taken by grab technique on hours 0, 24 and 96 in which approximately a total of 50 g of litter was collected from each of the four corners and the center of the plastic tub. These samples were combined and thoroughly mixed in a whirl-pak bag before analysis. Remaining litter was evenly spread to maintain a flat surface area.

Litter pH measurements were taken with a Fisher Scientific Accumet pH meter 50 (Denver Instrument Company, Bohemia, NY, USA). This was performed by adding 45 ml of distilled water to 5 g of litter and allowed to equilibrate for 1 hour. After which time, the litters' pH was measured. Litter moisture was obtained by taking 10 g of litter, and drying for 48 hours at 100° C. Using the weight loss between initial and dried litter the percent moisture was determine.

Ammonia measurements were taken immediately before sulfuric acid addition (time 0), then, 24, 48, 72 and 96 hours post treatment application. A Dräger Chip Measuring System (CMS) was used, with a plastic rectangular measurement tub (17 x 25 x 12 cm) attached to pump (Dräger Safety, Inc., Lubeck, Germany). The measurement tub was placed directly on the litter filled tub, and air was pumped through the ammonia detection machine for 60 seconds before measurements were taken. After each

measurement, fresh air was forced into the pump, by fanning the attached measurement tub, with the pump running, up and down four to five times.

A ten gram subsample of litter obtained from the 0, 24 and 96 hour samples was utilized for bacteria recovery. This subsample was placed in sterile filter bags with 90 ml of sterile Phosphate Buffered Saline (Difco™), and homogenized in an AES Laboratoire Stomacher for 60 s. Serial dilutions were made by taking 1 ml of this initial dilution and adding it to 9 ml of sterile Phosphate Buffered Saline, each dilution was vortexed for five seconds before sequential dilutions were made. For each dilution, 0.1 ml was spread plated, in duplicate, onto each of the following media types.

Media utilized were; Plate Count Agar (Difco™) for total aerobic bacteria; Anaerobic Agar (Difco™) for total anaerobic bacteria; MacConkey's Agar (Difco™) for *E. coli*. *Salmonella* recovery was performed by two methods, direct plating and enrichment. Direct plating was performed utilizing the methods described above for the other media types using Xylose-Lysine-Tergitol 4 agar (Difco™). Enrichments were performed in Tetrathionate Broth, Hajna (Difco™) for 48 hours, and then streaked onto XLT4. This step was performed to ensure that any sample with *Salmonella* concentrations below enumeration limits were not falsely identified as negative.

Aerobic bacteria, *Salmonella* and *E. coli* were incubated aerobically overnight at 37° C. While anaerobic bacteria were incubated in an atmosphere consisting of 90% N₂, 5% CO₂, and 5% H₂ overnight at 37° C.

Data analysis was performed with SAS 9.2, using the general linear model procedure at 0.05 level of significance (SAS Institute 2009). Cfu/g litter was transformed

from visual counts to \log_{10} , and litter moisture was arcsine transformed. Means with significant differences were separated using Tukey's Honestly Significant Difference test. Data were analyzed for effects due to application rate and time.

Results

Sulfuric acid

Sulfuric acid treated litter had lower ammonia levels than the control at, 24, 48, 72 and 96 hours ($P < 0.001$, Table 7). By 48 hours, the 27.21 L/92.9 m² application rate had lower ammonia levels than the 9.07 application rate, 1.3 and 3.8 pm respectively ($P < 0.001$). At the 72 and 96 hour sampling time there was no difference in ammonia levels between any of the sulfuric acid treatments ($P < 0.001$).

Bacterial data for total aerobe, anaerobe and *Salmonella* levels are presented in Table 8. Total aerobic bacteria counts were lower in litter that received 27.21 L/92.9 m² sulfuric acid, at 24 ($P < 0.001$) and 96 hours ($P = 0.004$). Twenty four hours after sulfuric acid treatment, total anaerobic bacteria were lowest in the 27.21 L/92.9 m² treatment (3.1 \log_{10} cfu/g litter) while the control was higher with 5.4 \log_{10} cfu/g litter. Sulfuric acid when applied at 9.07 and 18.14 L/92.9 m², had the highest anaerobic bacteria levels, with both having an average of 7.1 \log_{10} cfu/g litter. By 96 hours litter treated with the 27.21 L/92.9 m² application rate had lower anaerobic concentrations, 4.2 \log_{10} cfu/g litter, compared to the control and other treatments ($P < 0.001$).

No *Salmonella* was recovered before the addition of the *Salmonella* cocktail (0 hour, Table 8). At 24 and 96 hours, no *Salmonella* was recovered from sulfuric acid

treated litter regardless of sulfuric acid application rate, while control litter had 2.4 and 2.7 log₁₀ cfu/g litter, respectively ($P<0.001$).

The 24 hours control litter pH remained basic at 8.8, while treated litter pH decreased to 6.3, 4.8 and 2.1, for 9.07, 18.14, and 21.27 L/92.9 m² sulfuric acid treatment levels, respectively (Table 9). By 96 hours, control litter pH remained at 8.8, while treated litter pHs were 7.2, 5.7 and 2.3 for the increasing sulfuric acid application rates (9.07, 18.14, and 21.27 L/92.9 m²).

Observed moisture percent for litter treated with sulfuric acid are shown in Table 10. Litter moisture correlated with the application rate; litter receiving higher application rates containing more liquid had higher percentage of moisture.

Aluminum sulfate

Twenty-four hours after treatment, litter receiving aluminum sulfate had lower ammonia emissions than the control litter which received only *Salmonella* ($P<0.001$), this trend would continue until the conclusion of the experiment at 96 hours (Table 11). At 24 hours, ammonia levels in treated litter were: 16.7, 10.9 and 7.5 ppm. With increasing amounts of aluminum sulfate there was a decrease in ammonia. Ammonia measurement for the untreated litter was 100.3 ppm. Samples collected at the 48 hour period showed that ammonia emissions in the control litter decreased to 88.3 ppm; however litter treated with aluminum sulfate still had lower ammonia emissions, 15.4, 9.0 and 6.4 ppm, again with ammonia decreasing with increasing amount of aluminum sulfate ($P<0.001$).

Seventy two hours after treatment, ammonia emissions for litter treated with aluminum sulfate remained steady at 15.4, 7.4 and 6.0 ppm. At this time, the the control decreased

to 67 ppm, but this was still significantly higher than the treated litter ($P < 0.001$).

Ammonia measurements at 96 hours reflected those of the previous sampling times, with aluminum sulfate treated litter having significantly lower ammonia measurements than the untreated litter; 12.3, 6.6, 3.3 ppm at application levels 22.7, 45.4 or 68 kg/92.9 m² respectively and control at 46.3 ppm ($P < 0.001$). For the 24, 48, 72 and 96 hour sampling times there was no difference in ammonia levels between the three application rates.

At 24 hours, total aerobic bacteria were 9.3, 9.5, and 9.2 for treated litter and 9.6 log₁₀ cfu/g for the untreated control (Table 12). A decrease was observed at 96 hours in the 68 kg/92.9 m² treated litter. The mean aerobic bacteria concentration for the 68 kg/92.9 m² treated litter was 8.2 log₁₀ cfu/g, this was significantly less than both the 22.7 and 45.4 kg/92.9 m² treated litter (9.9 and 9.6 log₁₀ cfu/g). However, this was not less than the control which had 9.2 log₁₀ cfu/g ($P = 0.005$). Twenty four hours after treatment with aluminum sulfate, anaerobic bacteria concentrations for litter treated with either 22.7, 45.4 or 68 kg/92.9 m² of aluminum sulfate were; 5.7, 6.4 and 5.4 log₁₀ cfu/g, respectively. At this time the untreated litter had 5.5 log₁₀ cfu/g ($P = 0.28$). Anaerobic bacteria concentrations at 96 hours reflected those at 24 hours, with no observable differences between treated litter (5.6, 5.1 and 5.2 log₁₀ cfu/g) and untreated litter (4.9 log₁₀ cfu/g).

Salmonella results for aluminum sulfate were not as promising as the sulfuric acid results. Before addition of the 30 ml *Salmonella* cocktail no *Salmonella* was recovered from the litter (Table 12). At 24 hours 4.6 log₁₀ cfu/g of *Salmonella* was recovered from the untreated litter; while litter treated with aluminum sulfate at 22.7, 45.4 or 68 kg/92.9 m² application rates had *Salmonella* concentrations of 4.2, 4.3 and 3.9 log₁₀ cfu/g,

respectively ($P=0.894$). *Salmonella* numbers at 96 hours in litter treated with 45.4 or 68 kg/92.9 m² of aluminum sulfate were 1.7 log₁₀ cfu/g. Litter treated with the lowest application rate, 22.7 kg/92.9 m² had the highest concentration of *Salmonella*, with 3.9 log₁₀ cfu/g. Untreated litter was intermediate with 2.5 log₁₀ cfu/g. None of these were statistically different from each other ($P=0.162$).

Litter pH measurements (Table 13) before treatment with aluminum sulfate were all between 7.4 and 7.8, which is slightly more neutral than normal litter pH. Twenty four hours after treatment, a decrease was seen in 45.4 and 68 kg/92.9 m² application rates, 5.9 and 4.3, respectively. The pH for litter receiving aluminum sulfate at 22.7 kg/92.9 m² remained unchanged from time 0 at 7.5; while the pH for untreated litter increased to 8.8 ($P=0.023$). At 96 hours, pH values were almost unchanged from the 24 hour sampling with pHs of; 7.4, 5.9, 5.8 and 8.4 for the treatments 22.7, 45.4, 68 and 0 kg/92.9 m² respectively ($P<0.001$). The observed pH values for both the 45.4 and 68 kg/92.9 m² application rates were significantly lower than the pH values for the 22.7 and 0 kg/92.9 m² treated litter at 24 and 48 hours.

Percent litter moisture measurements are given in Table 14. No differences were observed after application of aluminum sulfate. The decreasing moisture in the untreated litter was the probable cause for the decreasing ammonia emissions.

Discussion

While several types of poultry litter amendments exist for ammonia reduction the most used and effective are acidifiers. These amendments work by reducing both litter pH; it does this by trapping ammonia as ammonium. This study investigated the

efficacy of sulfuric acid and aluminum sulfate at reducing ammonia levels and *Salmonella* levels in broiler litter. Previous research has shown that reduction in bacterial concentrations is greatest when a litter pH of 4 or less is achieved (Pope and Cherry 2000; Payne *et al.*, 2002; Payne *et al.*, 2007; Williams *et al.*, 2012).

In agreement with previous research, this experiment showed that as litter pH decreased below 4, a significant difference was observed in the amount of bacteria present. The highest application rate of sulfuric acid (21.27 L/92.9 m²) produced a litter pH of 2.1 and 2.3 at 24 and 96 hours, respectively. Coinciding with pH reduction an almost 4 log reduction was observed for total aerobic and anaerobic bacteria at 24 hours, and an approximate 2 log reduction at 96 hours. In regards to *Salmonella*, litter treated with sulfuric acid had no recoverable *Salmonella* at 24 and 96 hours.

Sulfuric acid worked extremely well at reducing litter ammonia levels regardless of application rate, with ammonia levels of <4 ppm at 24 hours reduced from over 200 ppm before treatment. These ammonia levels remained relatively unchanged for the duration of the experiment with only a slight fluctuation between sampling times. It is interesting to note that no difference was observed in ammonia levels between application rates even though differences were observed in litter pH. Sulfuric acid does pose a certain risk as it is very corrosive, must be handled with appropriate caution and training or certification.

Aluminum sulfate produced similar ammonia reduction results as sulfuric acid, and as with previous research the lower the litter pH the more effective an amendment is at trapping ammonia as ammonium. Treatment with aluminum sulfate reduced ammonia

to acceptable levels that would not impact broiler live performance (Fulton, 2008). Despite differences in litter pH, additional amounts of aluminum sulfate did result in observable lower amounts of ammonia. This would be helpful to the farmer as using less of the product would save money. Aluminum sulfate did not impact bacterial numbers as dramatically as sulfuric acid did. Only total aerobic bacteria were affected by the addition of 68 kg/92.9 m², the highest application rate used, and this was not observed until 96 hours after treatment. Aluminum sulfate had no effect on total anaerobic bacteria or *Salmonella*.

Overall, both aluminum sulfate and sulfuric acid were effective at reducing litter ammonia emissions. Sulfuric acid produced promising results for bacteria reduction, as it almost completely eliminated *Salmonella*, and had significant reduction of total aerobic and anaerobic bacteria. The last finding of interest is that higher amounts of either chemical did not result in lower ammonia emissions or lower bacteria numbers up to 96 hours after treatment.

References

- Batz, M. B., S. Hoffmann and J. G. Morris Jr. 2011. Ranking the risks: The 10 pathogen-food combinations with the greatest burden on public health. Emerging Pathogens Institute, University of Florida.
- Chinivasagam, H. N., M. Redding, G. Runger and P.J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. *British Poultry Science*. 51:311-318.
- Foster, J. W. and H. K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* 172: 771-778.
- Foster, J. W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. *Journal of Bacteriology*. 175:1981-1987.

- Fulton, R. M. 2008. Other toxins and poisons. Pages 1245 in Diseases of Poultry 12th Edition. Y. M. Said ed. Blackwell Publishing Professional. Ames, Iowa.
- Line, J. E. 2002. Campylobacter and *Salmonella* populations associated with chickens raised on acidified litter. Poultry Science. 81:1473-1477.
- Martin, S. A. and M. A. McCann. 1998. Microbiological survey of Georgia poultry litter. J. Appl. Poult. Res. 7:90-98.
- Payne, J. B., E. C. Kroger and S. E. Watkins. 2002. Evaluation of litter treatments on *Salmonella* recovery from poultry litter. J. Appl. Poult. Res. 11:239-243.
- Payne, J. B., J. A. Osborne, P. K. Jenkins and B. W. Sheldon. 2007. Modeling the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and water activity. Poult. Sci. 86:191-201.
- Pope, M. J. and T. E. Cherry. 2000. An evaluation of the presence of pathogen on broilers raised on Poultry Litter Treatment®-treated litter. Poult. Sci. 79:1351-1355.
- Rothrock Jr., M. J., K. L. Cook and J. G. Warren. 2010. Microbial mineralization of organic nitrogen forms in poultry litters. Journal of Environmental Quality. 39:1848-2010.
- SAS Institute. 2009. SAS User's Guide. Statistics. Version 9.2 Edition. SAS Inst. Inc, Cary, NC.
- Terzich, M., C. Quarles, M. A. Goodwin and J. Brown. 1998. Effect of poultry Litter Treatment® (SODIUM BISULFATE®) on death due to ascites in broilers. Avian Dis. 42:385-387.
- Volkova, V. V., R. H. Bailey and R. W. Willis. 2009. *Salmonella* in broiler litter and properties of soil at farm location. PLoS ONE. 4:6403.
- Williams, Z., J. P. Blake and K. S. Macklin. 2012. The effect of sodium bisulfate on *Salmonella* viability in broiler litter. Poult. Sci. In review.

Table 7. Average ammonia levels (ppm) for each treatment at 0, 24, 48, 72 and 96 hours after treatment with sulfuric acid.

Sulfuric Acid L/92.9 m ²	Time (Hours) Post Treatment				
	0	24	48	72	96
9.07	270	3.8 ^z	3.8 ^y	2.8 ^z	2.7 ^z
18.14	266	2.3 ^z	2.7 ^{yz}	2.6 ^z	2.4 ^z
27.21	225	1.4 ^z	1.3 ^z	0.9 ^z	1.1 ^z
Control	210	63.7 ^y	36.7 ^x	27.9 ^y	20.8 ^y
SEM	14.9	5.4	0.5	1.4	2.3
<i>P</i> Value	0.0530	<0.0001	<0.0001	<0.0001	0.0007

No difference was observed before application of sulfuric acid, 0 hours ($P=0.053$). At 24 hours, sulfuric acid treated litter had lower ammonia levels than the control regardless of application rate ($P<0.001$). No difference was observed between the sulfuric acid treated groups at 24 hours. At 48 hours sulfuric acid treated litter again had lower ammonia levels than untreated control. The highest application rate, 27.27 L/92.9 m², had lower ammonia levels than the 9.07 L/92.9 m² application rate ($P<0.001$). At 72 ($P<0.001$) and 96 ($P<0.001$) hours, sulfuric acid treated litter had significantly lower ammonia levels than the control, there was no difference between application rates at these times.

^{x-z}Numbers in the same column with different superscripts are significantly different ($P<0.05$).

SEM = Standard error of the mean

Table 8. Total aerobic, anaerobic and *Salmonella* concentrations at 0, 24 and 96 hours after treatment with sulfuric acid (log₁₀ cfu/g litter)

Sulfuric Acid L/92.9 m ²	Aerobic			Anaerobic			<i>Salmonella</i>		
	0	24	96	0	24	96	0	24	96
9.07	7.2	7.9 ^y	8.3 ^y	4.1 ^z	7.1 ^x	6.9 ^y	0	0 ^z	0 ^z
18.14	7.3	8.0 ^y	8.4 ^y	4.4 ^{yz}	7.1 ^x	7.3 ^y	0	0 ^z	0 ^z
27.21	7.3	4.3 ^z	6.0 ^z	4.5 ^y	3.1 ^z	4.2 ^z	0	0 ^z	0 ^z
Control	7.2	8.1 ^y	8.1 ^y	4.5 ^y	5.4 ^y	6.5 ^y	0	2.4 ^y	2.7 ^y
SEM	0.038	0.239	0.367	0.067	0.189	0.281	0	0.113	0.451
<i>P</i> Value	0.0879	<0.0001	0.0049	0.0120	<0.0001	0.0003	NS	<0.0001	0.0060

Reduction in bacterial numbers was observed for total aerobes and anaerobes for the 27.21 L/92.9 m² sulfuric acid application rate at 24 and 96 hours between is and the other three treatments. Interestingly the 24 hour anaerobe numbers increased in the 9.07 and 18.14 acid treated litter compared to the control ($P<0.001$) Reduction was observed for *Salmonella* at 24 and 96 hours due to application of sulfuric acid ($P<0.001$).

^{x-z}Numbers in the same column with different superscripts are significantly different ($P<0.05$).

SEM = Standard error of the mean

Table 9. Litter pH levels at 0, 24 and 96 hours after sulfuric acid application.

Sulfuric Acid L/92.9 m ²	Time (Hours) Post Treatment		
	0	24	96
9.07	8.90	6.33 ^y	7.17 ^x
18.14	8.89	4.78 ^y	5.73 ^y
27.21	8.89	2.07 ^z	2.31 ^z
Control	8.87	8.80 ^x	8.81 ^w
SEM	0.0007	0.268	0.194
<i>P</i> Value	0.0605	<0.0001	<0.0001

Significant differences in litter pH were observed at 24 and 96 hours. At 24 hours the 27.21 L/92.9 m² sulfuric acid application rate had the lowest litter pH, 2.1, and the 9.07 L/92.9 m² and 18.14 L/92.9 m² application rates were significantly higher with pHs of 6.3 and 4.8 respectively. The control litter pH remained unchanged at 8.8 ($P < 0.0001$) for the duration of the study. At 96 hours there was a significant difference between all treatments, the 27.21 L/92.9 m² had the lowest pH with 2.3, 18.14 L/92.9 m² had the next lowest at 5.7, while 9.07 L/92.9 m² was next with a pH of 7.2 ($P < 0.0001$).

^{w-z}Numbers in the same column with different superscripts are significantly different ($P < 0.05$).

SEM = Standard error of the mean

Table 10. Litter moisture (percent) for litter treated with sulfuric acid.

L/92.9 m ²	Time (Hours)		
	0	24	96
9.07	37.90	41.16 ^y	30.05 ^y
18.14	40.28	41.30 ^y	34.65 ^x
27.21	39.78	44.19 ^y	39.27 ^w
0	36.39	33.34 ^z	25.55 ^z
SEM	1.04	1.16	0.82
<i>P</i> Value	0.0965	0.0010	<0.0001

Percent litter moisture for litter treated with varying application rates of sulfuric acid. Litter moisture was affected according to the increasing amount of water needed to dilute the sulfuric acid before application. Decreasing moisture in the control would cause the decrease in observed ammonia levels.

^{w-z}Numbers in the same column with the different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean

Table 11. Ammonia measurements (ppm) for litter treated with 22.7, 45.4, 68 or 0 kg/92.9 m² of aluminum sulfate.

Application rate kg/92.9 m ²	Time (Hours) Post Treatment				
	0	24	48	72	96
22.7	195.3	16.7 ^z	15.4 ^z	15.4 ^z	12.3 ^z
45.4	283.7	10.9 ^z	9.0 ^z	7.4 ^z	6.6 ^z
68	250.3	7.5 ^z	6.4 ^z	6.0 ^z	3.3 ^z
Control	225.3	100.3 ^y	88.3 ^y	67.0 ^y	46.3 ^y
SEM	32.8	6.9	5.5	5.1	3.3
<i>P</i> Value	0.3379	<0.0001	<0.0001	<0.0001	<0.0001

After 24 hours a significant decrease was observed due to treatment with aluminum sulfate regardless of application rate, this trend would continue until the conclusion of the experiment at 96 hours. No differences were observed among the treated litter, while ammonia emissions in untreated litter (0 kg/92.9 m²) remained higher than any of the treated litter.

^{x-z}Numbers in the same column with difference superscripts are significantly different at

$P \leq 0.05$.

SEM = Standard error of the mean

Table 12. Total aerobic, anaerobic and Salmonella concentrations (\log_{10} cfu/g litter) at 0, 24, 48, 72 and 96 hours after treatment with sulfuric acid.

Application rate kg/92.9 m ²	Time (Hours) Post Treatment								
	Aerobic			Anaerobic			<i>Salmonella</i>		
	0	24	96	0	24	96	0	24	96
22.7	10.5 ^x	9.3	9.9 ^y	4.9 ^z	5.7	5.6	0	4.2	3.9
45.4	10.2 ^{xy}	9.5	9.6 ^y	5.9 ^{yz}	6.4	5.1	0	4.3	1.7
68	10.1 ^{yz}	9.2	8.2 ^z	6.2 ^y	5.4	5.2	0	3.9	1.7
Control	9.9 ^z	9.6	9.2 ^{yz}	5.8 ^{yz}	5.5	4.9	0	4.6	2.5
SEM	0.067	0.199	0.240	0.278	0.371	0.162	0	0.604	0.995
<i>P</i> Value	0.0022	0.6131	0.0045	0.0455	0.2805	0.0992	0	0.8941	0.4076

Ninety-six hours after treatment with 68 kg/92.9 m² of aluminum sulfate there was an observable difference in total aerobic bacteria, with this amount of aluminum sulfate resulting in less aerobic bacteria than the 22.7 and 45.4 kg/92.9 m² application rates ($P=0.005$). No other differences were seen for any bacteria enumerated due to treatment with aluminum sulfate.

^{x-z}Numbers in the same column with different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean

Table 13. Litter pH levels at 0, 24 and 96 hours after aluminum sulfate application.

Application rate kg/92.9 m ²	Time (Hours) Post Treatment		
	0	24	96
22.7	7.50 ^{yz}	7.46 ^{xy}	7.42 ^y
45.4	7.43 ^z	5.85 ^{yz}	5.99 ^z
68	7.54 ^{yz}	4.31 ^z	5.83 ^z
0	7.82 ^y	8.80 ^x	8.38 ^y
SEM	0.072	0.421	0.293
<i>P</i> value	0.0230	0.0003	0.0007

Twenty four hours after application with aluminum sulfate a significant decrease in litter pH was observed between treated litter and untreated litter. Litter receiving no aluminum sulfate had the highest pH of 8.8, while pH in litter treated with aluminum sulfate became more acidic with increasing amounts of aluminum sulfate; 7.5, 5.9 and 4.3. At 96 hours, pH in the untreated litter, and litter receiving 22.7 and 45.4 kg/92.9 m² of aluminum sulfate remained relatively unchanged; 8.4, 7.4 and 5.9, respectively; while litter pH for the 68 kg/92.9 m² application rate 1.5 points to 5.8.

^{x-z}Numbers with different superscripts are significantly different at $P \leq 0.05$.

SEM = standard error of the mean

Table 14. Litter moisture (percent) for litter treated with aluminum sulfate.

kg/92.9 m ²	Time (hours) post treatment		
	0	24	96
22.7	25.26 ^y	20.13	16.36
45.4	25.58 ^{yz}	19.96	15.61
68	23.89 ^{yz}	20.86	15.74
0	23.20 ^z	21.71	15.46
SEM	0.24	0.91	0.01
<i>P</i> value	0.0018	0.5436	0.5331

No difference was observed in litter moisture after application of aluminum sulfate. Decreasing ammonia emissions for the litter receiving no sulfuric acid can be attributed to the decreasing litter moisture.

y-zNumbers in the same column with different superscripts are significantly different at

$P \leq 0.05$.

SEM = Standard error of the mean

Reducing ammonia volatilization and *Salmonella enterica* concentrations in poultry litter using *Bacillus subtilis*

Abstract

An unintended waste product of intensive rearing conditions of broiler type chickens is litter; litter contains many bacteria including pathogens such as *Salmonella*. Other bacteria present in poultry litter produce ammonia as an end product of their metabolism. The need to control ammonia has led to the development of chemical litter amendments that trap ammonia. However, to control ammonia for a typical six week broiler flock grow-out, these amendments would have to be reapplied. *Bacillus subtilis* is known to utilize ammonia and produces subtilins that are effective at inhibiting the growth of other bacteria.

In this paper, a series of experiments utilizing several *B subtilis* strains are described. The *B. subtilis* chosen were based on *in vitro* inhibition of several *S. enterica* serovars and ammonia utilization. In experiment one, spores from two strains of *B. subtilis* were applied individually to litter at a rate of 10^7 or 10^9 spores per gram of litter. In experiment two, three strains of *B. subtilis* were mixed and applied to poultry litter at a rate of 10^7 cells, as either spores or vegetative cells. For both studies, a cocktail of five *Salmonella* serovars commonly associated with poultry was added to the litter. Ammonia measurements were taken before treatment and then at 24, 48, 72 and 96 hours post treatment. Litter moisture, pH and bacterial enumerations were performed before

treatment and then at 24 and 96 hours post treatment. In experiment one, no effect was observed on bacteria concentrations; however there was a decrease in ammonia production in litter treated with *B. subtilis* after 24 hours and this trend continued until 96 hours. Experiment 2 produced no differences in ammonia or bacteria levels for either spores or vegetative cells compared to their respective control ($P>0.05$). The data reflects the potential of *B. subtilis* at ammonia reduction, and that a single, direct application onto litter may not be effective at reducing *Salmonella*.

Introduction

In the United States, *Salmonella* is the leading cause of bacterial food-borne illness accounting for more than one million cases, with poultry meat products responsible for approximately 21% of the total (Batz *et al.*, 2011, Scallan *et al.*, 2011). *Salmonella*, if shed from colonized broiler chickens will, persist in the litter environment and can colonize sequential flocks if not eliminated. It was shown in a recent study that the incidence of farms with *Salmonella* positive poultry litter could be as high as 83% (Chinivagasam *et al.*, 2010).

A high level of ammonia in commercial broiler houses is a serious problem and is typically associated with wet litter and poor ventilation. The U.S occupational Safety and Health Administration has set an eight hour exposure limit to ammonia at 25 ppm. Ammonia at levels greater than 50 ppm will irritate mucus membranes around the eyes, trachea and upper respiratory tract. This irritation could lead to lesions in these areas, and eventually significant performance loss, if ammonia levels are not reduced (Fulton, 2008). Chemical litter amendments were introduced to help alleviate ammonia problems,

the most common type of these amendments are the acidifiers. These amendments work by lowering the pH of broiler litter to trap ammonia as ammonium salt (Terzich *et al.*, 1998). However, once the buffering capacity of the chemicals is depleted they must be reapplied, which can be costly to the farmer. In recent years, researchers have turned to biological sources, specifically bacteria, to reduce ammonia in broiler houses with the hope that one could be found that would utilize ammonia and persist in the poultry house indefinitely. *Bacillus subtilis*, a gram positive spore forming rod, is one such bacterium that can utilize ammonia as a source of nitrogen when no other source is readily available (Bergey's Manual 9th edition, 1994; Hall and MacVicar, 1955). *B. subtilis* also produces bacteriocins called subtilins, these proteins will inhibit the growth of other species of nearby bacteria, potentially *Salmonella*.

B. subtilis can be an effective way to reduce *Salmonella* on broiler carcasses and litter ammonia production if used as a diet supplement. Santoso *et al.* (1998) supplemented broiler and layer hen diets with 1 or 2% dried *B. subtilis*. A significant decrease in ammonia production was observed for both bird types, as well as 0.1 lbs of feed per lb of gain lower feed conversion. Direct inoculation of day old chicks with *B. subtilis* has also proven to be an effective method of reduction of *Salmonella* and *Clostridium perfringens* shedding in feces (La Ragione and Woodward, 2003). Direct feeding of *B. subtilis* spores to broilers has also been shown to reduce the incidence of *Salmonella* contamination of poultry carcasses. Fritts *et al.* (2000) found that half of the carcasses from birds fed *B. subtilis* had no recoverable *Salmonella*, while all the untreated control birds' carcasses were positive for *Salmonella* contamination.

The goal of these studies was to identify *B. subtilis* isolates that when directly applied to poultry litter; would reduce ammonia emissions, inhibit the growth of *Salmonella* spp., and persist longer than chemical amendments.

Materials and Methods

***Bacillus subtilis* preparation**

Experiment 1

Two strains of *B. subtilis* were chosen based on their ability to inhibit the growth of *Salmonella* spp. when grown in vitro via zone of inhibition measurement; these strains were identified as 01 and 301. A sufficient number of spores of both strains were obtained and stored in sterile phosphate buffered saline (PBS, Difco™, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) at 2° C until needed. In this trial, each strain was applied to litter at a rate of 10⁷ or 10⁹ spores per gram. Two controls were used in experiment; sodium bisulfate at a rate of 45.4 kg/92.9 m², and 50 ml sterile PBS.

Experiment 2

For this trial, six new isolates of *B. subtilis* were chosen (71, 183, 185, 206, 294 and 302), again based on their *in vitro* ability to inhibit *Salmonella* growth, and their ability to utilize ammonia in M9 minimal media (Difco™). These six strains were mixed and applied to the litter at a rate of 10⁷ cells per gram as either spores or vegetative cells. Spores were stored in PBS and vegetative cells were stored in tryptic soy broth (TSB,

Difco™) at 2° C until needed. For this experiment, a sterile PBS control was utilized for the spore cells and a sterile TSB control was used for the vegetative cells.

Experiments 1 and 2

Spore preparation

B. subtilis spores were obtained from freezer stock (-80° C) and inoculated into 3 ml of TSB; then incubated at 30° C for 24 hours. After incubation 0.1 ml of inoculated TSB was spread plated onto *Bacillus* sporulation agar plates and incubated for 5 days at 30° C. Following the second incubation spores were harvested using a sterile cotton swab and put into sterile milliQ water, this solution was then centrifuged at 10,000g for 5 minutes. After centrifugation, spores were transferred to a 100 ml centrifuge tube and resuspended in 50 ml of sterile milliQ water. Resuspended spores were heated at 80° C for 15 minutes. Finally spores were put into 50 ml of PBS and stored at 2° C until initiation of experiments.

Litter preparation

Approximately 50 kg of broiler chicken litter was obtained from the Auburn University Poultry Science Research Unit, and stored in a heated room (27° C). Before a trial was to be performed, three 10 g samples were taken and dried at 110 C for 48 hours to determine litter moisture. From these results, the amount of water to be added to increase litter moisture to 30% was determined. Seventy two hours before the initiation of the trial, water was added in small amounts while the litter was stirred to achieve an even distribution of water. The combination of moisture and heat would promote the ureolytic bacteria to produce ammonia.

From the 50 kg litter described above, 3 kg was put into plastic tubs (0.53 m x 0.39 m) and then allowed to equilibrate for one hour. After one hour, ammonia levels were measured using a Dräger Chip Measure System (CMS), with a plastic rectangular tub (17 x 25 x 12 cm) attached to the provided pump (Dräger Safety, Inc., Lubeck, Germany). Measurements were taken placing the inverted tub directly on the litter surface, the pump was allowed for 60 seconds and then measurements were taken. After each measurement the tub was purged with fresh air, by moving the tub up and down 4 – 5 times, allowing fresh air into the tub. Ammonia measurements were taken immediately before treatment was applied and then at 24, 48, 72 and 96 hours after treatment application.

Samples for laboratory analysis: bacterial enumeration, pH and moisture content, were taken by removing litter (a total of 50 g) from each of the four corners and center of each tub with gloved hand. This litter was then placed in a sterile whirl-pak bag and placed on ice for transport to the laboratory. These samples were taken before treatments and then at 24 and 96 hours post treatment.

Immediately prior to treatment with *B. subtilis*, a 30 ml of cocktail of five poultry associated *Salmonella enterica* serovars, 10^9 cfu/ml, was added to the litter. The *Salmonella* serovars chosen for these experiments were Enteritidis, Kentucky, Montevideo, Heidelberg and Typhimurium. Each of these was previously isolated from either commercial poultry processing plants or broiler farms and identified using 16S rRNA identification. Each isolate was then removed from storage at -80° C and streaked onto tryptic soy agar with 5% sheep red blood cells (BBL), incubated for 24 hours and then an isolated colony was selected and used to inoculate brain heart infusion broth

(Difco™). After 24 hour incubation, 6 ml of each serovar were added to together to constitute the 30 ml cocktail. This cocktail was applied by evenly distributing the entire 30 ml onto the surface of the litter and then thoroughly mixing into the litter using a gloved hand. Immediately after addition of the *Salmonella* cocktail, 50 ml of each *B. subtilis* treatment (experiment 1 and 2), sodium bisulfate at 45.4 kg/92.9 m² (experiment 1), 50 ml sterile water (experiment 1), 50 ml PBS or TSB (experiment 2), was added to the litter; each treatment was added to three tubs.

Sample Analysis

Bacterial enumeration was performed by weighing out 10 g from the initial samples, then placing the litter into whirl pak filter bags containing 90 ml sterile PBS and stomaching for 60 seconds (AES Laboratoire). After stomaching, serial 1:10 dilutions were made in sterile PBS. From these dilutions, 0.1 ml from the appropriate dilution was spread plated onto agar plates in duplicate.

Culture media used in these experiments were Plate Count Agar (Difco™), for total aerobic bacteria, Anaerobic Agar (Difco™) for anaerobic bacteria, MacConkey agar (Difco™) for *E. coli*, Tetrathionate broth, Hajna (Difco™) for enrichment of *Salmonella*, and Xylose Lysine Tergitol 4 (XLT4; Difco™) agar for detection and enumeration of *Salmonella*. *Salmonella*, *E. coli* and total aerobic bacteria were incubated aerobically at 37° C for 24 hours; anaerobic bacteria were incubated in an anaerobic atmosphere that consisted of 5% CO₂, 5% H₂, and 90% N₂. After 24 hours plates were removed from incubation and enumerated. *Salmonella* enrichment was performed in 10 ml of Tetrathionate broth, Hajna (Difco™), inoculated broth was incubated for 48 hours at 37°

C, with shaking. After enrichment, a single XLT4 plate was streaked for isolation and incubated at 37° C for 24 hours.

Litter pH was obtained by placing 5 g of litter into 45 ml of deionized water; this mixture was allowed to equilibrate for one hour. pH measurements were taken with a Fisher Scientific Accumet pH meter 50 (Denver Instrument Company, Bohemia, NY, USA). Moisture content was calculated by weighing 10 g litter which was then dried at 110° C for 48 hours. After drying, litter was reweighed and litter moisture was calculated.

Data analysis was performed using SAS 9.2, utilizing the general linear model (GLM) at the 0.05 level of significance (SAS Institute, 2009). Before GLM analysis bacteria counts were \log_{10} transformed and litter moisture percentages were arcsine transformed. Mean separation was achieved using Tukey's Honestly Significant Difference Test. Because of differences in starting ammonia levels, ammonia data was transformed into a percent reduction from initial levels, then arcsine transformed. Due to the fact that spore cells and vegetative cells were stored in either PBS or TSB, each cell type had its own control and differences were analyzed by contrasts at the 0.05 level of significance.

Results

Experiment 1

Ammonia

It was observed that *B. subtilis* isolate 01 at both application concentrations was effective at reducing ammonia emissions by 24 hours after application ($P < 0.001$, Table

15). Isolate 01 at 10^9 spores per gram would continue to be effective until the conclusion of the experiment at 96 hours ($P<0.001$). At 72 and 96 hours, isolate 01 at the lower application rate also proved effective at reducing ammonia levels ($P<0.001$).

Isolate 301 was not effective at reducing ammonia until 72 hours after treatment, and then a high concentration (10^9 spores/g litter) was needed to achieve ammonia reduction ($P<0.001$). This treatment combination would also prove sufficient to reduce ammonia at 96 hours. None of the isolate spore concentration combinations were as effective at reducing ammonia as sodium bisulfate until 96 hours, at which time ammonia reduction for isolate 01 was equal.

Bacteria

Only total aerobic bacteria were affected by the addition of *B. subtilis* spores, and this was an increase in concentration over the untreated control litter ($P=0.001$, Table 16). Treatment with *B. subtilis* had no effect on anaerobic bacteria or *Salmonella*, and no *E. coli* was recovered.

Experiment 2

Ammonia

The data indicates that when applied to poultry litter, the *B. subtilis* strains chosen for this experiment were ineffective at reducing ammonia (Table 17). The only observable reduction was seen at the 72 hour sampling time, due to the treatment with *B. subtilis* spores ($P=0.01$). This difference was gone at the 96 hour sampling time

($P=0.77$). After analysis, no differences were observed due to treatment with vegetative cells.

Mean concentrations of *Salmonella* are given in table 18, and as can be seen are not affected by treatment with either *B. subtilis* spores or vegetative cells ($P>0.05$). No differences were observed for total aerobic and anaerobic concentrations (Data not given). No differences were observed for litter pH, or moisture due to treatment with *B. subtilis* for either experiment (Data not shown).

Discussion

Despite positive results from the preliminary *in vitro* data, once applied to poultry litter, *B. subtilis* proved ineffective at reducing litter ammonia or inhibiting *Salmonella* growth. No isolate, application concentration, or cell stage combination had any observable effect on the bacteria present in poultry litter. This is in contrast to the results of previous work that showed significant reduction in *Salmonella*, both in fecal shedding and carcass contamination (La Ragione and Woodward, 2003; Fritts *et al.*, 2000). However, this reduction was the result using *B. subtilis* as a direct fed microbial or direct inoculation of the broilers, while the data presented in this paper results from the direct application of *B. subtilis* to poultry litter. This study shows that a single dose direct application of *B. subtilis* cells may not be an effective method of delivery of the bacterium into litter, with regards to ammonia control and *Salmonella* reduction. During *in vitro* testing, *B. subtilis* only interacted with *Salmonella*, but when applied to poultry litter there are hundreds of other bacteria species present. Because of the high numbers

of bacteria present, the probability that the *B. subtilis* would come into direct contact with *Salmonella* is low.

The ammonia results indicate that, of all possible treatment combinations performed; only isolate 01 could potentially work. While *B. subtilis* is effective at reducing ammonia as a direct fed microbial (Santoso *et al.*, 1998), the data presented here indicates that it's potential when applied directly to litter is low. It is known that *B. subtilis* can use ammonia as an intermediate for nitrate reduction when no other nitrogen source is available (Hall and MacVicar, 1995). However, in poultry litter there are other sources of nitrogen, and the amount of ammonia volatilizing from the litter could be more than the bacterium can metabolize.

In both experiments a decrease was observed in litter moisture, regardless of treatment. The decrease in moisture would be detrimental to the survival of *B. subtilis*, and may lead to sporulation, rendering the bacterium ineffective at reducing ammonia or *Salmonella*. Addition of moisture may help the bacterium function; however, addition of moisture would also facilitate ammonia production.

As a direct fed microbial new *B. subtilis* cells are constantly being added to the intestines whereas in this project the cells were applied only once. This would facilitate the action of *B. subtilis* simply by increasing the number of cells present, replacing dead cells, and replacing those spores that never germinated into vegetative cells. The goal of these studies was to identify a *B. subtilis* isolate that, through direct application, would reduce ammonia emissions and *Salmonella* concentrations. The data presented here shows that this application method of *B. subtilis* may not be an effective method of

delivery. Isolate 01 did show some effectiveness at reducing ammonia when used as a litter amendment, and its efficacy as a direct fed microbial will be assessed in future studies.

References

- Batz, M. B., S. Hoffmann and J. G. Morris Jr. 2011. Ranking the risks: The 10 pathogen-food combinations with the greatest burden on public health. Emerging Pathogens Institute, University of Florida.
- Bergey's Manual of Determinative Bacteriology 9th Edition. 1994. John G. Holt ed. Williams and Wilkins, Baltimore.
- Chinivasagam, H. N., M. Redding, G. Runger and P.J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. *Brit. Poultry Sci.* 51:311-318.
- Fritts, C. A., J. H. Kersey, M. A. Motl, E. C. Kroger, F. Yan, J. Si, Q. Jiang, M. M. Campos, A. L. Waldroup and P. W. Waldroup. 2000. *Bacillus subtilis* C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. *J. Appl. Poult. Res.* 9:149-155.
- Fulton, R. M. 2008. Other toxins and poisons: Ammonia. Page 1245 in *Diseases of Poultry* 12th edition. Y. M. Saif ed.
- Hall, L.M., and R. MacVicar. 1955. Ammonia as an intermediate in nitrate reduction by *Bacillus Subtilis*. *J. Biol. Chem.* 305-310.
- La Ragione, R. M. and M. J. Woodward. 2003. Competitive exclusions by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Vet. Microbiol.* 94:245-256.
- Santoso, U. S. Ohtani, K. Tanaka and M. Sakaida. 1998. Dried *Bacillus subtilis* culture reduced ammonia gas release in poultry house. *J. Anim. Sci.* 806-809.
- SAS Institute. 2009. SAS User's Guide. Statistics. Version 9.2 Edition. SAS Inst. Inc, Cary, NC.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis.* 17:7-15.
- Terzich, M., C. Quarles, M. A. Goodwin and J. Brown. 1998. Effect of Poultry Litter Treatment® (PLT®) on the development of respiratory tract lesions in broiler. *Avian Pathol.* 27:566-569.

Table 15. Ammonia reduction in percent from time 0 for experiment 1.

Isolate and application rate in spores/g litter	Time (Hours) post treatment			
	24	48	72	96
01 @ 10 ⁹	66 ^y	82 ^y	88 ^y	93 ^{xy}
01 @ 10 ⁷	65 ^y	75 ^{yz}	85 ^y	92 ^{xy}
301 @ 10 ⁹	56 ^{yz}	76 ^{yz}	85 ^y	90 ^y
301 @ 10 ⁷	42 ^z	68 ^z	74 ^z	86 ^z
Sodium Bisulfate	95 ^x	96 ^x	96 ^x	97 ^x
Sterile PBS (control)	51 ^z	71 ^z	71 ^z	85 ^z
SEM	4.3	2.6	17.3	1.3
<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001

At 24 hours isolate 01 at either application rate had better ammonia reduction than the control. Again at 48 hours isolate 01 at 10⁹ spores/g litter had better ammonia reduction than the control. At 72 hours isolate 301 at 10⁹ and isolate 01 at either application rate had better ammonia reduction percentages than the control. At 96 hours, isolate 01 at both concentrations and 301 at 10⁹ had ammonia reduction greater than the control. Also at 96 hours, ammonia reduction for isolate 01 applied at either concentration was equal to that of sodium bisulfate.

^{x-z}Numbers in the same column with different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean

Table 16. Bacteria enumeration results from experiment 1 (log₁₀ cfu/g litter).

Isolate and application rate in spores/g litter	Time (Hours) Post Treatment								
	Aerobic			Anaerobic			<i>Salmonella</i>		
	0	24	96	0	24	96	0	24	96
01 @ 10 ⁹	9.8 ^w	9.0	9.3 ^y	6.8 ^v	5.7	5.4	0	2.6	3.5
01 @ 10 ⁷	9.9 ^u	9.2	9.3 ^y	5.4 ^z	5.8	5.4	0	4.3	3.6
301 @ 10 ⁹	9.7 ^x	9.3	9.3 ^y	5.4 ^y	6	5.7	0	4.3	3.5
301 @ 10 ⁷	9.5 ^y	9.8	9.1 ^{yz}	7.1 ^u	6	5.2	0	4.1	3.7
SB	9.8 ^v	9.6	9.1 ^{yz}	5.6 ^w	6	5.6	0	4.3	3.6
Control	9.4 ^z	9.8	8.8 ^z	5.6 ^x	6.4	5.3	0	4.2	3.5
SEM	0.001	0.33	0.059	0.001	0.342	0.274	0	0.541	0.393
<i>P</i> Value	<0.0001	0.4789	0.0014	<0.0001	0.7784	0.7275	-	0.2288	0.9967

No reduction in bacteria type enumerated was observed, for any treatment at any sampling time. In fact, at 96 hours an increase was seen in the number of total aerobic bacteria for litter treated with *B. subtilis*.

^{y-z}Numbers with different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean

SB = sodium bisulfate

Table 17. Ammonia measurements in percent reduction from time 0 for Experiment 2.

Treatment	Time Hours Post Treatment			
	24	48	72	96
Spore cells	80.4	81.0	94.9 ^y	88.1
PBS	81.4	80.6	86.0 ^z	88.6
<i>P</i> Value	0.77	0.90	0.01	0.77
SEM	4.1	3.9	2.3	2.2
Vegetative cells	89.8	87.6	94.9	94.1
TSB	91.6	84.6	93.3	95.6
<i>P</i> Value	0.85	0.58	0.55	0.73
SEM	4.2	3.4	1.4	1.7

Percent reduction in litter ammonia measurements for experiment 2, *P* values are contrasts between the treatment and its control. *B. subtilis* spore or vegetative cells were applied to litter at 10^7 cells/g. PBS served as the control for spore cells and TSB served as the control for the vegetative cells. The only statistical difference was observed 72 hours after treatment. At this time the litter treated with spore cells had higher ammonia reduction than the control ($P=0.01$). However, at 96 hours there was no difference between the spore treated litter and the untreated control ($P=0.77$)

^{y-z}Numbers with different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean.

Table 18. Comparison results of *Salmonella* recovery from Experiment 2 (\log_{10} cfu/g litter).

Treatment	0	24	96
Spore			
Cells	0	5.3	3.8
PBS	0	5.2	3.8
<i>P</i> Value		0.459	0.893
SEM		0.8	0.2
Vegetative			
Cells	0	5.3	3.8
TSB	0	5.3	4.3
<i>P</i> Value		0.653	0.212
SEM		0.1	0.3

No differences were observed for *Salmonella* concentrations (\log_{10} cfu/g litter) in litter treated with *B. subtilis* spore cells or vegetative cells. The authors speculate that due to the differences in the litter environment versus *in vitro* testing that *B. subtilis* was ill equipped to inhibit *Salmonella* in the much harsher litter environment.

Numbers with different superscripts are significantly different at $P \leq 0.05$.

SEM = Standard error of the mean

Microbiological analysis of poultry litter using high throughput 16S rRNA sequencing

Abstract

Little is known about the microbial ecology of animal wastes, including poultry litter, other than data collected from growth media based culture methods. Accurately describing the entire bacterial community of animal wastes is an important step in learning how to control the bacterial populations present, namely pathogens, but also bacteria that produce harmful waste products such as ammonia. In this study 454 Pyrosequencing is used, with the goal of identifying the majority if not all of the bacteria present in poultry litter. After analysis 4 phylum of bacteria were identified, Actinobacteria (51.18%), Firmicutes (25.64%), Proteobacteria (10.06%) and Bacteroidetes (0.06%). A group of unknown or other bacteria comprised the remaining 13.06% of the sequences. Several important families of bacteria were identified including, Pseudomonadaceae (3.08%), Enterobacteriaceae (1.85%) and Staphylococcaceae (0.36%). These families contain potentially harmful bacteria such as *Pseudomonas*, *Salmonella*, *E. coli* and *Staphylococcus*. Being pathogens, these bacteria are the most commonly studied and used to describe the bacterial community in poultry litter. However, the data presented here shows that these bacteria make up approximately 5% of the total population. This study illustrates that a more thorough understanding of the bacteria community present in poultry litter is needed, so that current management

techniques can be improved upon to facilitate the control of the bacteria population with the aim of pathogen reduction, as well as reduction of the ammonia producing ureolytic bacteria.

Introduction

A by product of the intensive grow-out of commercial broiler flocks is litter. Litter contains the original material, in the southeast this is predominately pine shavings, along with excreta, feathers, feed, litter beetles, and bacteria. Total bacterial levels in poultry litter can reach 10^{11} CFU/g litter (Terzich *et al.*, 2000). Some bacteria that are commonly found in poultry litter of importance are *Salmonella*, *E. coli*, *Staphylococcus* spp., *Clostridium perfringens* and *Campylobacter* spp.. All of these bacteria can cause serious illnesses in humans, and a few of them can cause serious illness in chickens; resulting in performance loss and possibly mortality. These bacteria cause approximately 3.2 million cases of human food-borne illness a year (Scallan *et al.*, 2011). These bacteria can easily be grown and identified on culture agar. However, there are numerous bacteria that cannot be cultured as easily.

In recent years, with the advent of DNA sequencing, and molecular phylogenetic analysis, new bacteria have been discovered that previously were unknown. Direct comparisons of culture dependent and culture independent analyses often yield different results. Culture analysis of litter yielded only 3.3 positive for *Campylobacter*, while molecular analysis of same samples resulted in 31% positive samples (Bang *et al.*, 2002). Comparing the culture based and molecular results for *Campylobacter*, led the researchers to conclude that *Campylobacter* does not persist in the litter environment or

became viable but nonculturable. In agreement Lu *et al.* (2003) observed differences in enteric bacteria and *Staphylococcus* when using culture media and molecular methods. The researchers found through culture methods that, of the total bacterial population, enteric bacteria made up 0.1% and *Staphylococcus* made up 13%. However, based on molecular analysis, enteric bacteria comprised 2% of the population, and *Staphylococcus* 8%.

Through molecular methods several new groups of bacteria have been identified in poultry litter. The first of these is a group of wood degrading bacteria normally found in the intestines of termites (Lu *et al.*, 2003). The second is a group of ammonia producing bacteria (Rothrock *et al.*, 2008). The data shows that culture methods alone may not be adequate to describe the entire bacteria population present. While culture based methods are quicker, and less costly, culture dependent methods are more labor intensive and cannot target all bacteria present like molecular methods. In contrast molecular methods, such as pyrosequencing, take longer to perform and have a much higher cost than traditional culture methods.

Pyrosequencing is a relatively new method of DNA sequencing. It is based on the release of pyrophosphate during elongation of new DNA strands (Nyren *et al.*, 1987). When a new nucleotide is attached to the DNA strand, a pyrophosphate molecule is released; luciferase is then attached to the pyrophosphate by ATP sulfurylase and the light emitted by the luciferase can be measured in real time (Ronaghi *et al.*, 1996).

Pyrosequencing has been used to sample wounds, soils, intestines and many other environments. Analysis of soils using this technique yielded up to 55,000 individual

bacteria sequences (Roesch *et al.*, 2007). A survey of the bacterial assemblage of wounds from several patients, by culture and 454 pyrosequencing, found 44 bacterial families through 16S rRNA analysis and only nine were found through culture methods (Price *et al.*, 2009). The results from both studies allowed the researchers to quickly analyze and compare the differences in the bacterial communities caused by different soil management techniques or different antibiotic treatments.

Pyrosequence analysis of the intestines of twenty cows revealed 274 different species of bacteria in 142 genera (Dowd *et al.*, 2008). Sequence analysis yielded several thousand individual sequences per samples; this allowed species of bacteria that made up less than 0.1% of the total population to be detected.

The goal of this project was to use 454 pyrosequencing to analyze the entire bacterial community of poultry litter. Previous molecular analysis of poultry litter used primers for specific bacteria, namely pathogens, and used these to identify the bacteria present (Bang *et al.*, 2002; Lu *et al.*, 2003; Rothrock *et al.*, 2008). 454 pyrosequencing performed in this paper utilized universal eubacteria primers, 347 forward and 803 reverse (Nossa *et al.*, 2010). These primers target a region of the 16S rRNA gene and allow for the sequencing and identification of the majority of bacteria present. Pyrosequencing analysis has not been performed on poultry litter, and should give better insights into the complete bacterial community present.

Materials and Methods

Litter Sampling

Litter samples were taken on a six house commercial broiler farm. Samples were taken from three houses by scraping the heel of a gloved hand across the top 5.08 cm of litter and then placing it into a sterile whirl pak bag. Samples were collected from three locations in the house; exhaust fans, middle near the control room, and evaporative cooling cells. Litter was collected from under the feed and water lines at each location. Collected samples from each location were thoroughly mixed and kept on ice during transport to laboratory.

DNA Extraction

Upon arrival at the laboratory DNA was extracted from each litter sample using the E.Z.N.A.[®] Stool DNA Kit, according to instructions provided (Omega bio-tek, Norcross, GA, USA). First, 250 mg of sample, 200 mg of glass beads and 540 μ L of SLB buffer were vortexed for 10 minutes in a 1.5 mL centrifuge tube. After vortexing 60 μ L of DS buffer and 20 μ L of proteinase K were added, vortexed for 5 s, and incubated at 70° C for 10 minutes. After incubation, 200 μ L of SP2 buffer was added; samples were vortexed for 30 seconds and then incubated on ice for 5 minutes. Upon removal from ice, samples were centrifuged at 13,000 rpm for 5 minutes. After centrifugation, 400 μ L of supernatant was aspirated into a new 1.5 mL centrifuge tube. To this tube, 200 μ L of HTR reagent was added, then vortexed for 10 seconds and incubated at room temperature (22° C) for 2 minutes. Next, samples were centrifuged at 13,000 g for 2 minutes, and 250 μ L of supernatant was aspirated into a new 1.5 mL centrifuge tube. To this new tube,

250 μ L of BL buffer and 250 μ L of absolute (100%) ethanol were added and subsequently vortexed for 10 s. The entire solution was then transferred to a HiBind DNA Column, centrifuged for 1 minute at 13,000 g, after which the flow through liquid was discarded. To the HiBind DNA Column, 500 μ L of Buffer VHB was added; after addition of buffer the column was centrifuged at 10,000 g for 30 seconds, again flow through was discarded. Next DNA was washed by adding 700 μ L of DNA wash buffer to the column; column was then centrifuged for 1 minute at 13,000 g. Wash step was performed twice, after washing column was transferred to a new 1.5 mL micro centrifuge tube. Elution buffer (100 μ L) was added to the column, incubated at room temperature (22°C) for 2 minutes and then centrifuged for 1 minute at 13,000 g. Extracted DNA was stored at -20 °C until needed.

PCR Conditions

PCR amplification was performed in a Bio-rad iCycler iQ5 Multicolor Real-time PCR Detection System (Bio-rad, Hercules, CA, USA). Initial DNA denaturation was performed at 94° C for 2 minutes. Following initial denaturation, a touchdown PCR was performed with 15 cycles consisting of 94° C for 30 s, 65° C for 30 s (decreasing by 1° C every cycle), and 72° C for 30 s. The second stage of the PCR consisted of 20 cycles of 94° C for 30 s, 50° C for 30 s and 72° C for 30 s. A final elongation was performed at 72° C for 7 minutes. After PCR reaction was finished, amplified DNA was held at 4° C and then stored at -20° C.

Primers used for eubacterial 16S rRNA amplification were 347F (5'-GGAGGCAGCATRRGGAAT) and 803R (5'-CTACCRGGTATCTAATCC) developed

by Nossa *et al.* (2010). These primers would generate PCR amplicons of 454 base pairs, needed for pyrosequencing.

After PCR amplification, samples were run on a 1% agarose gel, with a 1 kb ladder standard. Bands were excised using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). Agarose bands were excised using a sterile scalpel and placed into a 1.5 ml micro centrifuge tube. Agarose gel band was weighed, and 10 μ l of Capture Buffer Type 3 was added per 10 mg of gel. Agarose gel and capture buffer were mixed by inversion and incubated at 60°C until the gel was completely dissolved. Samples were then transferred to an assembled GFX Microspin Column and collection tube. After transfer, samples were incubated at room temperature (22° C) for 1 minute. The assembly was then centrifuged at 16,000 g for 30 s. Following centrifugation, flow through liquid was discarded. Next, 500 μ l of Wash Buffer Type 1 was added to the GFX Microspin Column. Column was again centrifuged at 16,000 g for 30 s. Samples were washed twice to ensure purity. After second wash, collection tube was discarded and GFX Microspin Column was placed in a sterile 1.5 ml micro centrifuge tube. Twenty micro liters of Elution buffer type 6 was added to the center of the GFX Microspin Column filter, and assembled column and 1.5 ml microcentrifuge tube were centrifuged at 16,000 g for 1 minute. Purified DNA was stored at -20° C.

Purified DNA was quantified using the Nanodrop 1000 (Thermo Fisher Scientific). Fifteen samples were chosen and pooled in equimolar concentrations (16 ng/ μ l) and sequenced at Engencore (Columbia, SC) utilizing the Roche 454 FLX sequencer with Titanium chemistry.

Sequence Analysis

Sequences were analyzed utilizing the Quantitative Insights into Microbial Ecology (QIIME) sequence analysis software (Caporaso *et al.*, 2010). Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity. The Ribosomal Database project within QIIME was used to align sequences utilizing the PyNast procedure, and then sequences were grouped using a complete linkage clustering method. Clusters were formed using uclust, which forms clusters based on percent sequence identity. Representative sequences from each OTU were selected and taxonomic identity was assigned to each sequence using the RDP taxonomic classifier with 90% confidence (Wang *et al.*, 2007).

Results

After sequence analysis bacteria were classified into 4 known phyla; the Actinobacteria (51.18%), Firmicutes (25.645%), Proteobacteria (10.06%) and Bacteroidetes (0.06%). The remaining 13.06% of the sequences were grouped as “other” or unknown.

In the Actinobacteria phylum, 7 families were identified; Brevibacteriaceae, Dermabacateraceae, Bifidobacteriaceae, Corynebacteriaceae, Yaniellaceae, Glycomycetaceae, and Dietziaceae, and a family of unknown Actinomycetales. The second most abundant phylum the Firmicutes, contained 3 known families; the Ruminococcaceae, Bacillaceae, Staphylococcaceae, the phylum also contained 4 unknown families in the groups Bacillales, Bacilli, Clostridia, and Clostridiales. The Proteobacteria phylum consisted of; Pseudomonadaceae, Enterobacteriaceae,

Moraxellaceae, Xanthomonadaceae, Desulfovibrionaceae, and an unknown family of Oceanospirillales. The Bacteroidetes, were classified into a single family the Bacteroidaceae. A complete breakdown of relative abundances of each family are shown in Table 19.

Discussion

454 Pyrosequencing analysis of the bacterial population of poultry litter, revealed a diverse community of bacteria. The predominant phylum was the high G+C content, gram positive Actinobacteria, accounting for half of the total sequences. Firmicutes, another gram positive bacteria phylum constituted almost one fourth of the sequences. Unlike the Actinobacteria, Firmicutes are characterized as having a low G+C content. In agreement, previous research identified 82% of the total bacterial sequences as being gram positive bacteria (Lu *et al.*, 2003). However, this previous sequence analysis revealed that most of the bacteria present (62%) were low G+C content bacteria, while the current study found the majority of the bacteria to have high G+C content. Farm location, differences in both broiler and litter management techniques, feed formulations, could all attribute to the differences observed.

Proteobacteria, a phylum of gram negative bacteria, made up 10% of the sequences identified; this phylum contains many of the pathogens associated with human food-borne illnesses, including *E. coli*, *Salmonella*, *Vibrio* and *Pseudomonas*. Two classes of Proteobacteria were identified, the Gammaproteobacteria and Deltaproteobacteria. At the Family level of taxonomic organization, two important

families of Gammaproteobacteria were found, the Pseudomonadaceae and the Enterobacteriaceae, in relative abundances of 3.08 and 1.85% respectively.

In disagreement with previous research Staphylococcaceae comprised less than 1% of the total bacterial sequences. Culture methods have observed *Staphylococcus* in concentrations of greater than $8 \log_{10}$ cfu/g litter (Terzich *et al.*, 2000; Williams *et al.*, 2012). In agreement, Lu *et al.* (2003) found a decrease in the relative abundance of *Staphylococcus* present, 13% by culture method and 8% by sequence analysis. This difference could be attributed to the inability of growth media to grow all bacteria that may be present in poultry litter. This disparity, illustrates the difference in methods and the possible over estimation of bacteria present by culture dependent methods.

The predominant family of bacteria present in poultry litter, Ruminococcaceae, is a family of starch and cellulose degrading bacteria (Duncan *et al.*, 2007). This bacteria family is likely degrading the cellulose present in the pine shavings as well as starches in spilt feed. The Ruminococcaceae family made up almost one fourth of the bacterial sequences. This finding is in agreement with Stanley *et al.* (2012), who also found Ruminococcaceae in high relative abundance in broiler litter.

A significant percentage of the bacterial sequences being identified as “other” is not surprising, and is in agreement with other 454 pyrosequencing analysis of litter that identified 11% of the sequences as unknown or other (Stanley *et al.*, 2012). The most logical explanation for this is that the sequences contained in the databases used for analysis are mainly of human origin. It would be entirely plausible that poultry being fed

an entirely different diet and living in a different environment would have a different intestinal microbial community.

The data presented shows the potential inadequacy of traditional culture dependent methods for describing the bacterial ecology of poultry litter. According to the data, bacteria that are normally investigated through culture dependent methods, Enterobacteriaceae, Staphylococcaceae and Pseudomonadaceae, constitute less than 10% of the bacterial community. A better understanding of poultry litter bacterial community is needed to determine any fluctuations in the relative abundance of the bacteria caused by different management techniques. This knowledge would facilitate the development of management techniques that would potentially select for beneficial bacteria, and inhibit the growth of not only pathogenic bacteria, but also ureolytic bacteria.

References

- Bang, D.D., A. Wedderkopp, K. Pedersen and M. Madsen. 2002. Rapid PCR using nested primers of the 16S rRNA and the hippuricase (hipO) genes to detect *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. *Mol. Cell. Probe.* 16:359-369.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez-Pea A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods.* 7:335–336.
- Dowd, S. E., T. R. Gallaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort and T. S. Edrington. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rRNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiology.* 8:125-132.
- Duncan, S. H., P. Louis and H. J. Flint. 2007. Cultivable bacteria diversity from the human colon. *Lett. Appl. Microbiol.* 44:343-350.

- Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16s rRNA and functional gene markers. *Appl. Environ. Microb.* 69(2):901-908.
- Nossa, C. W., W. E. Oberdorf, L. Yang, J. A. Aas, B. J. Paster, T. Z. DeSantix, E. L. Brodie, D. Malamud, M. A. Poles and Z. Pei. 2010. Design fo 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. *World J. Gastroenterol.* 16:4135-4144.
- Nyren, P. 1987. Enzymatic method for continuous monitoring of DNA polymerase activity. *Anal. Biochem.* 167:235-238.
- Price, L. B., C. M. Liu, J. H. Melendez, Y. M. Frankel, D. Engelthaler, M. Aziz, J. Bowers, R. Rattray, J. Ravel, C. Kingsley, P. S. Keim, G. S. Lazarus and J. M. Zenilman. 2009. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS One.* 4:1-10.
- Roesch, L. F. W., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *International Society for Microbial Ecology Journal.* 1:283-290.
- Ronaghi, M., A. Karamohamed, B. Petterson, M. Uhlen and Pal Nyren. 1996. Realtime DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242:84-89.
- Rothrock Jr., M. J., K. L. Cook, J. G. Warren and K. Sistani. 2008. The effect of alum addition on microbial communities in poultry litter. *Poult. Sci.* 87:1493-1503.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis.* 17:7-15.
- Stanley, D., M. S. Geier, R. J. Hughes and R. J. Moore. 2012. The role of gastrointestinal tract microbiota in chicken productivity. *Aust. Poult. Sci. Symp.* 23:262-265.
- Terzich, M., M. J. Pope, T. E. Cherry and J. Hollinger. 2000. Survey of pathogens in poultry litter in the United States. *J. Appl. Poult. Sci.* 9:287-291.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73:5261– 5267.

Table 19. Relative abundance of each family of bacteria in poultry litter.

Phylum	Class	Order	Family	Percentage
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	22.67
	Clostridia	Clostridiales	Other	0.27
	Clostridia	Other	Other	0.27
	Bacilli	Bacillales	Bacillaceae	0.59
	Bacilli	Bacillales	Staphylococcaceae	0.36
	Bacilli	Bacillales	Other	1.07
	Bacilli	Other	Other	0.40
Total				25.63
Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	0.14
	Actinobacteria	Actinomycetales	Brevibacteriaceae	21.59
	Actinobacteria	Actinomycetales	Dermabacteraceae	4.71
	Actinobacteria	Actinomycetales	Corynebacteriaceae	0.40
	Actinobacteria	Actinomycetales	Yaniellaceae	0.22
	Actinobacteria	Actinomycetales	Glycomycetaceae	0.14
	Actinobacteria	Actinomycetales	Other	22.62
	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	1.37
Total				51.19
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	3.08
	Gammaproteobacteria	Pseudomonadales	Moracellaceae	1.54
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	1.37
	Gammaproteobacteria	Oceanospirillales	Other	0.14
	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	1.10
	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	0.49
	Gammaproteobacteria	Other	Other	1.88
Total				9.6
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	0.06
Total				0.06

Relative abundance of bacteria 16S rRNA sequences present in poultry litter, based on 454 Pyrosequencing analysis. Sequences were analyzed utilizing the QIIME software, and OTUs were formed with 97% sequence similarity.