Evaluating Litter Sampling Methodology and the Persistence of Nontyphoidal *Salmonella* in Reused Poultry Litter

By

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Abstract

Nontyphoidal *Salmonella* is responsible for a significant proportion of foodborne illness in the United States and is prevalent in broiler houses all over the world. The study of environmental detection methods, management techniques to reduce prevalence, and understanding how *Salmonella* spp. colonize the GI tracts of broilers is important to establish an understanding of effective measures to control *Salmonella* spp. in broiler houses. *Salmonella* Enteritidis (SE) and *Salmonella* Kentucky (SK) are two widely prevalent serovars isolated in poultry production. Chapter 3 (C3) aimed to compare litter sampling methods in two experiments (C3E1, C3E2) with high (C3E1) and moderate (C3E2) environmental SE prevalence. Methods evaluated included litter grab (LG), drag swabs (DS), roller swabs (RS), and boot covers (BC). Both experiments for S1 involved spreading 800 birds evenly over 32 pens (25 birds/pen). The objective of Chapter 4 (C4) was to examine the effect of untreated control (UC), probiotic blend (PB), sodium bisulfate (SB), sodium formate (SF), and Windrow Compost (WC) treatments on the persistence of SE in broiler houses by using LG, BC, and collecting ceca of market age birds. S2 consisted of four experiments (C4E1-4) with C4E1-C4E3 involving spreading 1000 birds equally over 40 pens (25 birds/pen) and S2E4 involving spreading 1200 over 48 pens due to the addition of 8 fresh bedding (FB) pens. In both C3 and C4 litter sampling was performed between flocks after challenging the first flock with 10^7cfu/ml at 6 days of age, with each experiment referring to the number of the flock reared in the same pens. The objective of Chapter 5 (C5) was to observe the effects of challenging chicks with 10^7cfu of SE and/or SK on the colonization of the ceca by either serovar by collecting the ceca at Day 14, 21, 28, 35, 42, and 49 of the first flock (C5E1) and during the second unchallenged flock (C5E2). Treatments in C5 involved challenging with SE before SK (SE/SK), SK before SE (SK), SK only, and SE only challenges. Prevalence data from C3 and C4 were analyzed by chi-squared test or Fisher's exact test when

applicable and differences were observed when $P \leq 0.05$. *Salmonella* counts observed in C4 and C5 were analyzed by GLM with differences observed when $P \leq 0.05$ and means were separated with Tukey's HSD. In C3E1 it was observed that RS were less sensitive in SE detection versus other methods ($P<0.0001$). However, in C3E2 SE detection using RS was similar in detection to LG, greater than DS ($P = 0.022$), and lower than BC ($P < 0.0001$). In C4E1-4 there were no differences observed between treatments in BC detection or cecal colonization although in C4E2 differences were observed in LG sampling between SF and PB ($P=0.043$) and SB ($P=0.001$). In C4E1 differences in SE colonization were observed between SE and SK/SE ($P=0.44$) and differences in SK colonization were observed between SK and SE/SK ($P=0.01$). Observations in C3 helped further establish environmental detection methods and the potential use of RS for research pens due to the practicality of use. The findings of C4 lead to the potential application of SF as an antimicrobial litter treatment. Additionally, data from C5 demonstrated that the introduction of one serovar of *Salmonella* spp. negatively affects the colonization of the subsequently introduced serovar in the GI tract of broilers. These findings make significant contributions to the growing body of knowledge concerning the control of *Salmonella* spp. in broiler houses.

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List of Abbreviations

BC	Boot Cover
C	degrees Celsius
μL	microliter
BHIB	brain heart infusion broth
CDC	Centers for Disease Control and Prevention
Cm	centimeters
CE	competitive exclusion
cfu	colony-forming unit
D	day
DNA	deoxyribonucleic acid
DS	Drag Swab
et al.	et alia (and others)
FAO	Food and Agriculture Organization of the United Nations
FB	Fresh Bedding
FDA	Food and Drug Administration
GI	gastrointestinal
GLM	general linear model
h	hour
HSD	honest significant difference
LG	Litter Grab
m	meter
mL	milliliter
NT	non-typhoidal
PB	Probiotic Blend
PBS	Phosphate buffered saline
PCA	plate count agar
RS	Roller Swab

s	second
S	*Salmonella*
SB	Sodium Bisulfate
SF	Sodium Formate salts
SE	*Salmonella* Enteritidis
SK	*Salmonella* Kentucky
Spp.	species
TTB	tetrathionate broth
UC	Untreated Control
US	United States
USDA	United States Department of Agriculture
XLT4	xylose lysine tergitol-4
WC	Windrow Composted

Chapter 1: Introduction

The domestic and worldwide trends of the increased consumption of poultry products poses challenges for meeting the demand for product while keeping consumers safe from foodborne illness. The United States Centers for Disease Control and Prevention (CDC) estimates that nontyphoidal *Salmonella* causes over 1.35 million cases of foodborne illness and 420 deaths yearly in the United States (CDC, 2019a). With poultry products accounting for a considerable proportion of outbreaks of foodborne disease (CDC, 2021) efforts to control this pathogen are expanding to the preharvest level. Farm-to-fork food safety practices are being emphasized to gain control over *Salmonella* and other relevant foodborne pathogens. The broiler house is a notable stage in production for horizontal transmission of *Salmonella*, resulting in contamination that can be carried to processing facilities. Although the hatchery has been identified as a critical source of infection of broiler chicks, the broiler house is a critical step in integration where infected seeder birds can spread *Salmonella* to other birds of the flock (Bailey, 1987). The shedding of *Salmonella* in the feces of colonized birds results in broiler litter being a reservoir for *Salmonella* in broiler houses. Reduction of the contamination of *Salmonella* in the litter can help prevent the colonization of the GI tract of susceptible broiler chicks placed on reused litter (Milner and Shafer 1952), limit horizontal transmission within the flock, and limit the contamination of the carcass and transport equipment before processing (Bailey et al., 2001). In-house windrow composting has been shown as an effective litter management technique to reduce *Salmonella* in broiler litter (Macklin et al., 2006, 2008). However, this technique has not been evaluated in its effect on the spread of *Salmonella* spp. from one flock to the next after litter is seeded with *Salmonella* spp. The further investigation of this and other litter management methods and their ability to reduce the contamination of litter, minimize horizontal transmission, and prevent the colonization of *Salmonella* of market age birds would serve as a great

contribution to the body of knowledge of farm-to-fork food safety and to the practices of the poultry industry.

Chapter 2: Review of Literature

2.1 General *Salmonella* characteristics

The characteristics of *Salmonella* spp. and its ubiquity provides a foundation to understand some of the challenges involved with controlling *Salmonella* spp. in not only poultry production but in the food production chain as a whole. *Salmonella* spp. is a resilient bacterium that infects many warm-blooded animals and is considered native microflora in some avian species. *Salmonella* spp. is a gram-negative, facultative anaerobic, mesophilic, and rod-shaped bacterium with peritrichous flagella that belongs to the Enterobacteriaceae family (Swayne et al, 2013). Typical phenotypic characteristics observed in *Salmonella* spp. include oxidase negative, catalase positive, hydrogen sulfide producing, glucose fermenting, and reduction of nitrate to nitrite (Grimont et al., 2000). Resistance to bile salts, tergitol, selenite, and novobiocin by *Salmonella* spp. allows for the selective isolation of this bacterium (Giannella, 1996). *Salmonella* spp. is an intracellular pathogen that is acid adapted and motile, allowing for this bacterium to move throughout the digestive tract and replicate within host-cells. The asymptomatic infection of some warm-blooded animals with *Salmonella* spp. poses problems in detecting *Salmonella* spp. in poultry production.

2.2 *Salmonella* Classification

Salmonella spp. received its name from Daniel Salmon, the superior of Theobald Smith, who isolated *Salmonella* spp. from pigs (Grimont, 2000). The genus *Salmonella* is further divided into two species and six subspecies; *Salmonella enterica* and *Salmonella bongori* which are further divided into subspecies *Salmonella enterica* subspecies *enterica*, *Salmonella enterica* subspecies *salamae*, *Salmonella enterica* subspecies *arizonae*, *Salmonella enterica* subspecies

diarizonae, *Salmonella* enterica subspecies *houtenae* and *Salmonella enterica* subspecies *Indica* (Reeves et al., 1989). The subspecies *S. enterica* subspecies *enterica* is comprised of more than 2579 serovars (Grimont & Weill, 2007). *Salmonella* is classified by its three surface antigens: the flagellar H antigen, the somatic O antigen, and the superficial Vi antigen (Giannella, 1996). Serogroups A, B, C1, C2, D and E are the most common O antigen serogroups in *Salmonella enterica* subspecies *enterica* and these serogroups cause the majority of *Salmonella* infections in warm blooded animals (approximately 99%) (Brenner et al., 2000; Popoff & Le Minor, 1997).

2.3 Salmonellosis in Humans

Salmonella spp. infection or Salmonellosis can cause several syndromes in humans including typhoid fever, focal infections, gastroenteritis, and septicemia (Giannella, 1996). *Salmonella* spp. is additionally referred to by its clinical manifestations in humans. Typhoid *Salmonella* (i.e. *Salmonella typhi* and *Salmonella paratyphi*) causes typhoid fever and Non-typhoid (NT) *Salmonella* primarily causes gastroenteritis in humans although extraintestinal infection is possible. Typhoid fever is transmitted by contaminated water or the fecal-oral route and is primarily a concern in developing countries. However, NT *Salmonella* is primarily consumed in food products in all parts of the world and was the leading cause of death and hospitalizations in the United States in 2017 among foodborne illnesses (CDC, 2019).

The consumption of contaminated foods can result in the colonization of the ileum and colon with NT *Salmonella*, although the acidic pH of the stomach in healthy individuals is lethal to *Salmonella* spp. alone (Giannella, 1996). Waterman and Small (1998) observed that the infectious dose for NT *Salmonella* is lower when it is present in a food source and NT *Salmonella* was more likely to survive if the surface of the food inoculated had a pH >3 or if inoculated foods were high protein. Additionally, the inability to produce adequate stomach acid

has been associated with increased risk of NT *Salmonella* infection (Howden & Hunt, 1987). The pathogenesis of *Salmonella* spp. relies on virulence factors, including the ability to replicate intracellularly, invade cells, produce toxins, flagella, type III secretion systems and the lipopolysaccharide coat although serovars differ in pathogenicity (Giannella, 1996; Jajere, 2019). Type III secretion systems, which are encoded in *Salmonella* Pathogenicity Islands (SPI) within the genome (Marcus et al., 2000; Sabbagh et al., 2010), are a crucial part of invading intestinal epithelium. These multichannel proteins inject effectors into intestinal epithelia, which triggers a signal transduction pathway resulting in 'ruffling' of the epithelial cell (Eng et al., 2015), which results in *Salmonella* being engulfed by epithelial cells in a similar manner as phagocytosis (Takaya et al., 2003). Additionally, Type III secretion systems also allow *Salmonella* spp. to reside in vacuoles undetected by injecting effectors into the vacuole that prevents fusion with the lysosome (Eng et al., 2015). The invasion of the intestinal epithelium and lymphoid follicles results in multiplication of *Salmonella* spp. that can result in invasion of the mesenteric lymph nodes. The ability of *Salmonella* spp. to survive within macrophages allows it to move through the reticuloendothelial system (Monack et al., 2004). Most infections in healthy individuals are limited to gastroenteritis although the liver, spleen, gallbladder, and other organs may be infected depending on immune response and serovar (Giannella, 1996). The immunocompromised, elderly, and infants are at increased risk of extraintestinal NT *Salmonella* infection. The colonization of the intestinal epithelium and secretion of toxins usually result in an acute inflammatory response of the gastrointestinal (GI) tract that may manifest as ulceration of the GI tract and/or symptoms of cramping, diarrhea, and fever that usually start within 6-48 hrs upon ingestion of contaminated foods and can last between 2-7 days (Giannella, 1996; Santos et al., 2003).

Complications with controlling typhoid fever include the ability of typhoidal *Salmonella* to remain in a patient that has recovered from typhoid fever in a 'carrier state' (Levine et al., 1982). This carrier state can result in the shed of typhoidal *Salmonella* in the feces due to the ability of typhoidal *Salmonella* to colonize the gallbladder after bypassing the intestinal epithelial barrier (Gonzalez-Escobedo et al., 2011). Although uncommon, it is possible for NT *Salmonella* to be shed from patients that have recovered from Salmonellosis for weeks after recovery (Sirinavin et al., 2004). Additionally, the use of antibiotics has been reported to not improve the duration of the carrier state (Buchwald & Blaser, 1984). The carrier state provides further challenges in controlling *Salmonella* spp. and allows for continual transmission of this bacterium if proper hygiene is not maintained.

2.4 Nontyphoidal *Salmonella* in Poultry Production

Poultry meat and eggs are often implicated as reservoirs for the transmission of NT *Salmonella* to humans. Poultry meat is now the most consumed meat per capita in the United States which resulted from declines in beef consumption from 97 pounds in 1999 to 83 pounds per capita in 2020, increases in chicken consumption from 89 pounds per person in 1999 to 112 pounds in 2020, and pork consumption remaining constant (Kuck and Schnitkey, 2021). In 2017, NT *Salmonella* caused 29% of 395 single pathogen outbreaks and chicken third highest food group in outbreak related illnesses and was associated with 23 single food related outbreaks (CDC, 2019b). The increase of the consumption of poultry products not only puts pressure on the industry to produce enough product to meet demand, but also challenges the industry to mitigate the risk of foodborne illness that may result from an increase in consumption of poultry. The trends in increased production may also result in higher incidences of Salmonellosis if significant improvements cannot be made in controlling NT *Salmonella.*

2.4.1 *Salmonella* in Broiler Breeder Houses

The ability of some serovars of *Salmonella* spp. to infect the ovaries and oviducts of broiler breeders allows for the transmission of *Salmonella* spp. through the shells and yolks of eggs (Gast & Beard, 1990; Gast et al., 2004; Gantois et al., 2008). Consequently, the serovars that are transmitted the most readily to the egg (*Salmonella* Enteritidis and *Salmonella* Typhimurium) are also the top two serovars isolated in confirmed cases in Salmonellosis in the United States (Gantois et al., 2008; CDC, 2016). The vertical transmission of some *Salmonella* spp. provides great challenge in controlling transmission from broiler breeder houses to the hatchery. Additionally, most NT *Salmonella* does not cause clinical manifestations in poultry, which makes it unlikely to detect the infection of a broiler breeder flock (Allen-Vercoe & Woodward, 1999; Andino et al., 2015). The spread of NT *Salmonella* horizontally can lead to flock infection that results in vertical transmission or the persistence of NT *Salmonella* in the environment that results from the shedding of this bacterium in the feces. Broiler breeders can become infected with NT *Salmonella* through the oral or cloacal uptake of contaminated litter or from breathing dust that was created from contaminated litter (Barrow, 1991; Nakamura et al., 1995; Gast et al., 1998; Bailey et al., 2006; Chadwick et al., 2020; Pal et al., 2021). In addition to vertical transmission of *Salmonella* spp. to the inner contents of eggs, it has also been observed that some *Salmonella* spp. can penetrate the shell membrane post-lay (Miyamoto et al., 1998; De Reu et al., 2006), which makes the control of NT *Salmonella* crucial in the environment of broiler breeding facilities. Strict biosecurity measures similar to those employed in broiler houses should be adhered to in order to control *Salmonella* spp. in these environments. The incidence of vertical and horizontal transmission of *Salmonella* spp. at broiler breeding facilities can cause

problems with contamination at the hatchery, which can cause further lead to contamination in broiler houses, processing facilities, and in meat products.

2.4.2 *Salmonella* in the Hatchery

The majority of poultry meat production is vertically integrated from the broiler breeding houses to hatcheries, then further to broiler houses, and then to the processing, packaging, and/or marketing of products. *Salmonella* can spread from one step in the production chain to the others, but the hatchery has been identified as a critical point of contamination of broiler chicks (Bailey, 1987). It has been observed that the prevalence of *Salmonella* spp. in hatchery transport pads is higher than in the environment of broiler houses, further implicating the hatchery as a primary source of contamination (Bailey et al., 2001). Additionally, it has been observed that chicks younger in age are colonized by significantly lower doses of NT *Salmonella* than older birds (Milner and Shafer, 1952). The ability of some serovars to infect the ovaries and oviducts of broiler breeders allows for the transmission of *Salmonella* spp. to young broilers through vertical transmission, which results in placing infected chicks in broiler houses (Gast & Beard, 1990; Gast et al., 2004; Gantois et al., 2008). The contamination of eggshells has also been observed as a source of horizontal transmission of *Salmonella* spp. to chicks in hatching cabinets (Bailey et al., 1992, 1994). Serovars found on the carcasses of processed birds have been matched to serovars recovered from the environment of hatcheries and from chick papers by some groups (Goren et al., 1988; Bailey et al., 2001), further fortifying the hatchery as the origin of *Salmonella* spp. contamination and spreading to later steps in production. The observed contamination at hatcheries and the ability of *Salmonella* spp. to readily colonize the GI tract of young chicks makes the control of *Salmonella* spp. at or before the hatchery a priority for the poultry industry.

2.4.3 *Salmonella* in Broiler Houses

The infected broiler chick can be referred to as a 'seeder bird' due to the incidence of infected birds spreading *Salmonella* spp. horizontally throughout a broiler flock by the shedding of *Salmonella* spp. in their feces. The result of this shedding is the contamination of broiler chickens which can lead to the further spread of *Salmonella* spp. within a flock and to subsequent flocks that are raised on the same litter. *Salmonella* spp. can be spread in broiler houses by contaminated litter being taken up orally or ingesting contaminated litter through the cloaca, or by breathing in contaminated poultry litter dust. Challenging birds orally, intratracheally, or by aerosolizing contaminated dust has been performed to support of these models of horizontal transmission of *Salmonella* spp. through contaminated litter (Barrow, 1991; Nakamura et al., 1995; Gast et al., 1998; Bailey et al., 2006; Chadwick et al., 2020; Pal et al, 2021). *Salmonella* is a resilient and adaptive bacterium and persists in litter for months to years (Davies & Wray, 1996a), which allows for transmission between flocks when reusing litter. Additionally, *Salmonella* spp. persisting in litter can contaminate market age birds and/or transport equipment (Bailey et al., 2001), potentially causing cross contamination during transport (Rigby et al., 1980) and/or processing. Colonization of the GI tract of market age birds with *Salmonella* spp. can create contamination problems at processing by shedding of *Salmonella* in the feces during transport (Rigby et al., 1980) to slaughter. Additionally, contamination of the carcass or GI tract of birds can contaminate defeathering equipment (Allen et al., 2003), other carcasses (Mulder et al., 1978) or scalding water at processing (Slavik et al., 1995) which can lead to further issues with cross contamination. In addition to birds becoming infected with *Salmonella* spp. from the environment, they can also become colonized by *Salmonella* spp. that contaminates poultry feed. The ability of *Salmonella* spp. to spread

horizontally in broiler flocks and ability to persist in feed, litter, dust, and on equipment makes management methods to mitigate contamination in broiler houses crucial in limiting *Salmonella* spp. contamination at processing.

2.5 *Salmonella* Enteritidis

Salmonella enterica subspecies *enterica* serovar Enteritidis (SE) is a widely prevalent serovar of NT *Salmonella* in poultry meat and egg products that commonly causes gastroenteritis in consumers. SE was the most frequent serovar isolated from 16.8% of culture confirmed cases of *Salmonella* spp. reported by the Laboratory-Based Enteric Disease Surveillance System in 2016 (CDC, 2016). The incidence of cases of foodborne illness caused by SE increased dramatically in the 1980s and became the predominant serovar isolated from humans in 1994 (Patrick et al., 2004).

It has been reported by some groups that SE has taken place of the ecological niche of *Salmonella* Gallinarum and *Salmonella* Pullorum since the early 20th century after these serovars were severely reduced due to control efforts (Rabsch et al., 2000; Foley et al., 2011). Like *Salmonella* Gallinarum, SE is classified into the group D1 serogroup with similar O antigen characteristics (Brenner and McWhorter, 1998). An additional similarity is that SE can be transmitted from the oviduct of layers to the surface and internal contents of eggs, which may have allowed SE to fill in the ecological niche of *Salmonella* Pullorum of Fowl Typhoid (Gast & Beard, 1990; Gast et al., 2004). When comparing NT *Salmonella* serovars, it has been observed that SE is transmitted to the albumen of the egg more readily than other serovars (Gantois et al., 2008; Wales & Davies, 2011). Typically, unlike fowl typhoid, there are no clinical manifestations of SE infection in birds, which provides challenges in detection of this serovar (Allen-Vercoe and Woodward, 1999).

The zoonotic transmission of SE to consumers of poultry products is not limited to egg products, although SE is considered a common contaminant of eggs. The carcass of chickens may become infected with SE, which is readily transmitted horizontally through broiler flocks (Gast and Holt, 1999). Additionally, this serovar is frequently detected in poultry farms (Velasquez et al., 2018). In 2014, SE was one of the top isolated serovars from carcass rinses of young chickens (USDA FSIS, 2014). In 2015 the CDC reported an outbreak of SE linked to raw, frozen, stuffed, and breaded chicken entrees resulting in 15 confirmed cases in 7 states resulting in 4 hospitalizations with the four isolated strains of SE observed to be resistant to ampicillin and tetracycline (CDC, 2021). An additional outbreak of SE was seen in similar products in August of 2021 resulting in 28 people becoming infected with the outbreak strain in 8 different states with 11 of those infected hospitalized, approximately 59,000 pounds of frozen product was recalled (CDC, 2021).

2.6 *Salmonella* Kentucky

Salmonella enterica subspecies *enterica* serovar Kentucky (SK) is a NT *Salmonella* serovar that falls within the C serogroup and is rising in prevalence in the United States. In the United States, SK was found to be the most frequently isolated serovar in carcass rinses in 2014 (USDA FSIS, 2014). However, SK was only isolated from 0.14% of cases of confirmed cases of clinical human disease in 2016 (CDC, 2016). Additionally, it has been observed that some SK subtypes can cause Salmonellosis in some cases although SK is not considered pathogenic to humans (Rauch et al, 2018). SK isolates have been observed to have antibiotic resistance genes for ciprofloxacin, chloramphenicol, sulfisoxasole, streptomycin, tetracycline, and others (Weill et al., 2006; Melendez et al., 2010; Diarra et al., 2014). Data from the National Antimicrobial Resistance Monitoring System in 2020 shows that 79% of *Salmonella* Kentucky isolates from

683 poultry samples were resistant to Streptomycin, 52.6% were resistant to tetracycline, and less than 2% were found to be resistant to other antibiotics (FDA, 2021). The antibiotic resistance patterns of SK do cause some concerns in the case that SK could adapt to become more host-adapted and pathogenic for humans in the future or potentially transfer antibiotic resistance genes to other serovars. The high prevalence of SK in carcasses of processed birds could cause major food safety issues if SK were to become more host adapted for humans, so the control of SK is still something to be considered by food safety specialists, poultry industry, and in agencies governing food safety.

2.7 Methods of Control

2.7.1 Biosecurity

The utilization of general hygienic practices and biosecurity is a necessity to reduce the prevalence of *Salmonella* spp. in all steps of poultry production. Stocking broiler breeders that are *Salmonella* spp. free is a crucial starting point in mitigating contamination of poultry products (van Immerseel et al., 2009), however, these efforts are useless if *Salmonella* spp. cannot be kept out of the production environment. The reintroduction of *Salmonella* spp. by workers makes the use of designated footwear between houses, foot baths, hand hygiene, and protective equipment important for decreasing cross contamination between houses and contamination of the houses from the environment (van Immerseel et al., 2009). Additionally, monitoring and/or restricting movement of visitors, personnel, and equipment to and from the farm can also limit cross contamination between facilities or contamination from the environment (van Immerseel et al., 2009). Implementing simple biosecurity and hygiene procedures correctly are important measures necessary to mitigate horizontal transmission of *Salmonella* spp. and should be commonly practiced procedures in all steps of production.

2.7.2 Biocontainment

The introduction of *Salmonella* spp. into the environment by pests is possible with cockroaches, litter beetles, maggots, red mites, and rats or mice acting as vectors (Henzler and Opitz, 1992; Davies and Ray, 1995a, 1995b; Moro et al., 2007; Kopanic et al., 2014). Additionally, flies have been observed to carry *Salmonella* spp. (Bailey, 2001), although their role as vectors is not well defined. These pests can come into direct contact with birds, contaminate feed, or contaminate objects that come in to contact with birds which creates multiple routes of exposure of birds to *Salmonella.* Mice are especially important vectors because they often harbor organ invasive *Salmonella* in the liver and GI tract, which results in shedding *Salmonella* spp. in the feces (Henzler and Opitz, 1992; Davies and Ray 1995b). Three-week old broilers have become infected with SE resulting from exposure to rat feces of rats experimentally infected with SE 2-5 months prior (Davies and Ray 1995b). Efforts to control rodents has shown decreases in SE prevalence in broiler breeder houses and laying hen houses (Davies and Ray, 1995b; Henzler et al., 1998). Measures to control pests include controlling the access of pests to buildings, placing bait station or traps, use of rodenticides or pesticides, and clearing the surrounding areas of vegetation or places for pests to inhabit are necessary to mitigate contamination (van Immerseel et al., 2009). These observations highlight the importance of biocontainment as an important management practice that is crucial in controlling the prevalence of *Salmonella* spp. in all steps of production.

2.7.3 Sanitation

Cleaning and disinfection of equipment is crucial in reducing the spread of *Salmonella* from house to house or from one area of production to another. According to Morgan-Jones, an effective cleaning regiment includes: 1) Dry cleaning to remove dirt followed by wet cleaning of

surfaces with detergent; 2) disinfection to kill micro-organisms; 3) rinsing to clear residues of disinfectants and 4) fumigation (Morgan-Jones, 1987). The efficacy of cleaning depends on correct use of products and following an adequate cleaning procedure (Davies and Breslin, 2003). These procedures are important because *Salmonella* spp. has been isolated from water lines, egg belts, egg collectors, feed hoppers, feeders, fan blades, transport cages, and even dirt near the entrances of broiler houses (Rigby et al., 1980; Bailey et al., 2001; Jones et al., 1995). Proper sanitation procedures have been observed to decrease the prevalence of *Salmonella* in broiler houses (Garber et al., 2003). Formaldehyde and phenols have been observed to be effective disinfectants for *Salmonella* spp. (Davies & Wray, 1995c; Davies et al., 2001). Additionally, chlorination of drinking water has shown some reduction of *Salmonella* spp. contamination (Poppe et al., 1986), and is important due to the ability of *Salmonella* spp. to contaminate ground water, irrigation water, and other water sources (Kovačić et al., 2017; Liu et al., 2018). Hydrogen peroxide, UV light, and ozone have been used to disinfect hatchery cabinets and the use of hydrogen peroxide and ozone reduced the number of *Salmonella* positive chicks (Bailey et al., 1996). Mitigating *Salmonella* spp. spread through poultry production is possible with the utilization of proper cleaning and sanitation method if they are enforced regularly and without deviation from effective practices, however, these practices are only a piece of the puzzle in reducing *Salmonella* spp. contamination of food products.

2.7.4 Litter Management

Litter is a known reservoir for *Salmonella* spp. and other foodborne pathogens in poultry houses, and therefore some management techniques can be utilized to mitigate the horizontal transmission of *Salmonella* spp. and other foodborne pathogens within or between flocks. It is a common practice in the United States and other countries to reuse litter in broiler houses for

multiple grow outs due to this practice being less labor intensive and more economical than full clean outs. This raises some concerns for the hygiene of broiler houses due to the ability of *Salmonella* spp. to persist in broiler litter for months to years (Davies and Wray, 1996a). However, the reuse of litter has been observed to reduce the spread of *Salmonella* spp. in broiler flocks which is most likely due to the competing microflora in the litter (Olesiuk, 1971). The prevalence of *Salmonella* spp. has also been observed to decrease as litter is reused (Roll et al., 2011). The reuse of poultry litter in the United States makes litter management practices important not only to reduce contamination with foodborne pathogens, but also practices are necessary to manage the conditions of the litter to optimize flock performance and welfare.

In addition to managing litter to reduce contamination with foodborne pathogens, farmers also must manage litter conditions such as ammonia emissions, which can negatively affect flock performance and welfare (Miles et al., 2004; Reece et al., 1980). Acidifying litter with Sodium Bisulfate ($NaHSO_4$) or Aluminum Sulfate ($Al_2(SO4)_3$) reduces ammonia levels and emissions in built up litter and is a commonly utilized litter management technique (Burgess et al., 1998). Although altering litter pH is necessary for ammonia control which leads to optimal broiler performance and welfare, these conditions also affect the microbial composition of litter. Litter acidification methods are not necessarily intended to manage the microflora of the litter, however they have been observed to reduce total aerobic bacteria and *Escherichia coli* counts in litter (Pope & Cherry, 2000). However, the reduction of *Salmonella* spp. has not been observed with the use of litter acidification methods and lowering the pH may be somewhat advantageous for *Salmonella* spp. persistence (Pope & Cherry, 2000; Williams et al., 2012). Additionally, Williams et al. (2012) saw no effect on total aerobic bacteria when using litter acidification methods but saw higher loads of *E. coli* with litter acidification use.

The management of litter moisture is also a concern of farmers in the broiler industry. Litter moisture influences aerobic bacteria, enteric bacteria, or *Salmonella* spp. survivability (Turnbull & Snoeyenbos, 1973; Carr et al., 1995; Payne et al., 2007; Dunlop et al., 2016). However, the management of litter moisture is not solely based on the reduction of foodborne pathogens. Improvements in performance have been observed with lower litter moisture and bacterial load that resulted from varying stocking densities (Jayalakshmi et al., 2009). Additionally, litter moisture that is too dry can dehydrate chicks and cause respiratory issues in the flock (Ritz et al., 2005). Ventilation in broiler houses can be adjusted to optimize litter moisture content and the relative humidity of the house (Valentine, 1964; Carr & Nicholson, 1980; Weaver & Meijerhof, 1991). Higher levels of ventilation between 0.047 to 0.422m^3/s have been observed to increase bird weight gain (Carr & Nicholson, 1980). Additionally, litter with 45% relative humidity was reported to increase performance, decrease litter moisture, and decrease ammonia burns on the feet and breasts of broiler chickens when compared to 75% relative humidity (Weaver & Meijerhof, 1991). Management practices concerning litter moisture, relative humidity, and ventilation are not only important in controlling the microbial load of litter but are also crucial in ensuring optimal flock performance and welfare.

An additional management technique that poultry farmers can utilize to improve litter quality is in-house windrow composting, which is performed by pushing litter into long piles running the length of the broiler house. This method has been observed to reduce litter moisture content while also reducing aerobic, anaerobic, coliform, *Salmonella* spp., *Clostridium perfringens*, and *Campylobacter* counts after 7 days of composting (Hartel et al., 2000; Macklin et al., 2006, 2008). Additionally, covering composted piles with a non-breathable polymesh tarp has been observed to reduce aerobic and anaerobic bacterial counts more effectively than

uncovered composting (Macklin et al., 2006). Reduction of the bacterial load of litter is accomplished by maintaining core temperatures greater than 50°C for at least 24 hours, therefore implementing a pasteurization effect against bacteria, yeasts, and molds. Turning the piles after a few days can reintroduce oxygen to microbes further breaking down organic materials and can help achieve homogenous pasteurization (Macklin et al., 2006). This method is effective in reducing the bacterial load of litter, managing litter moisture content, is low-cost, and is less labor intensive than performing full house clean outs between broiler flocks. However, this method does not ensure that all broilers are *Salmonella* spp. free at harvest, but it does help manage litter before placing susceptible chicks on reused litter.

2.7.5 Vaccination

Inducing immune resistance of breeders and laying hens to *Salmonella* spp. is an additional management practice utilized to control *Salmonella* spp. Vaccination of hens with attenuated or inactivated vaccines is a common practice, although it can only reduce susceptibility to *Salmonella* spp., not prevent colonization (Gast, 2007). The primarily targeted serovars with vaccination are *Salmonella* Typhimurium and SE due to their known pathogenicity in humans and prevalence in poultry production (CDC, 2016). The vaccination of hens with avirulent *Salmonella* Typhimurium has shown increases in long lasting antibodies in hens and progeny showed higher antibody response to *Salmonella* spp. challenge than unchallenged chicks (Hassan & Curtiss, 1996). Some vaccines have shown promise in reducing colonization of the reproductive organs and GI tract of laying hens after SE challenge, which reduces the transmission of SE to the egg (Gantois et al., 2006; Arnold et al., 2014). Additionally, it has been reported that vaccinated breeder flocks have lower prevalence of *Salmonella* spp. in the ceca and reproductive tracts in addition to lower prevalence in chicks, the environment of broilers reared

by vaccinated breeders, and in birds sent to processing (Dorea et al., 2010). However, other groups have seen that vaccination does not reduce the shedding of *Salmonella* spp. in hens, which highlights the importance of sanitation of the environment (Arnold et al., 2014; Sharma et al,, 2018). These observations lead to the importance of vaccination of hens and broiler breeders, but although this is commonly practiced,*Salmonella* spp. continues to persist in hatcheries, broiler houses, and causes foodborne illness in consumers because vaccines are only effective against the serovars they were developed for and are not administered by all farmers. Vaccines are an important piece of the puzzle in controlling *Salmonella* spp., however, proper sanitation and hygiene is still of the utmost importance.

2.7.7 Competitive Exclusion

Methods for the exclusion of *Salmonella* spp. from the GI tract of chicks have been investigated thoroughly due to the ability of *Salmonella* spp. to readily colonize the GI tract of young chicks (Milner and Shafer, 1952). Competitive exclusion can be explained as bacteria with similar niches competing for resources and/or receptors in a host (Fisher & Nehta, 2013), The use of probiotics are often proposed as a means to exclude bacteria causing foodborne illness in poultry, such as *Salmonella* spp. An early method investigated by Nurmi et al. (1973) involved introducing adult chicken gut contents or competitive exclusion (CE) to the crops of chicks to exclude the colonization of *Salmonella infantis* after challenge, which was successful in reducing *Salmonella* colonization of the crop, small intestine, and ceca. More defined cultures of CE have been shown to exclude SE colonization of the GI tract and other important foodborne pathogens (Nisbet et al., 2002). These observations lead to the development of a product called Aviaguard, which is a freeze-dried fermentation product that can be applied as a spray or in drinking water (Nakamura et al., 2002). This product was observed to reduce the shedding of SE

and *Salmonella* Typhimurium in the feces of 14-day old broilers (Nakamura et al., 2002). Zhang et al. (2007) observed that the administration of *Lactobacillus salivarius* and *Streptococcus cristatus* reduced the colonization of chicks with SE, SK, and *Salmonella* Typhimurim. The administration of different species of *Bacillus* spores have been observed to significantly reduce of colonization and shedding of SE in the GI tract (La Ragione and Woodward, 2003; Vilà et al. 2009). The concept of competitive exclusion of *Salmonella* spp. from the GI tract of chicks has been examined and shows some promise of reducing the colonization of *Salmonella* spp. However, these methods do not necessarily prevent colonization of *Salmonella* spp. and will not prevent *Salmonella* spp. from entering broiler houses from the hatchery or other sources of cross contamination.

Employing probiotic blends as a litter treatment is a relatively uninvestigated application of the competitive exclusion concept. De Cesare et al. (2019) observed a decrease in total aerobic and *Enterobacteriaceae* counts after employing the use of a *Bacillus* probiotic blend as a litter treatment. However, the application of probiotics to reduce the prevalence of foodborne pathogens has not been examined. The further use of probiotics for application in the environment is warranted. The observation that used bedding mitigates the spread of *Salmonella* spp. in broiler houses verus using fresh bedding suggests some proof of the exclusion of *Salmonella* spp. in the environment by other bacteria (Olesiuk, 1971). Advancements in probiotic use in the environment could be of value in sanitation and hygiene efforts in the poultry industry.

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The use of Roller Swabs for *Salmonella* Detection in Poultry Litter

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Roller Swab Litter Sampling for *Salmonella*

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SUMMARY

Litter sampling is utilized as a non-invasive and practical method to determine broiler flock *Salmonella* status. The common methods include boot cover/sock **(BC)**, drag swab **(DS)**, or litter grab sampling **(LG)**. Roller swabs are a new research method that can be used to sample litter without entering research pens. This study aimed to assess the use of roller swabs **(RS)** for *Salmonella* **(S)** detection in comparison to BC, DS, and LG. For Experiment 1, litter was sampled for two weeks following a broiler flock that was challenged at 6 days of age with $1x10^7$cfu of a nalidixic acid resistant strain of SE to establish a high litter prevalence of SE. In Experiment 2, sampling occurred after a subsequent flock was raised on the same litter. In Experiment 1, S was detected by RS less frequently (81%) than DS (95%), BC (97%), and LG (98%) ($P < 0.0001$). In Experiment 2, S detection using RS (23%) was similar to LG (17%), higher than DS (6%, $P = 0.022$), and lower than BC (55%, $P < 0.0001$). Although RS were a less sensitive sampling method when the litter prevalence of S was high, RS were equivalent to or better than LG and DS methods when S prevalence was low. The use of roller swabs allows for sampling of litter without entering each pen and has the potential to be utilized for *Salmonella* detection in research pen trials.

DESCRIPTION OF PROBLEM

Nontyphoidal *Salmonella* remains a common cause of foodborne illness in the United States, causing an estimated 1.2 million illnesses, more than 23,000 hospitalizations, and 450 deaths per year (CDC, 2016). In 2016, 53 state and regional public health laboratories reported 46,623 cases of culture-confirmed *Salmonella* infections to the Laboratory-Based Enteric Disease Surveillance System with *Salmonella* Enteritidis being the most frequently reported serotype (CDC, 2016). Litter sampling has been employed to determine poultry flock *Salmonella*

status since the 1980's (Kingston, 1981) and these methods are currently employed in the European Union in order to control *Salmonella* at the processing level by determining processing destination accordingly. Environmental sampling allows for a noninvasive, cost effective, and practical way for detection of *Salmonella*. The earliest method for litter sampling involves taking litter from random areas of a poultry house and forming a composite sample to directly culture, which is referred to as litter grab sampling **(LG)**. Another method utilized is drag swab sampling **(DS)**, which was developed by Kingston (Kingston, 1981) and was improved by pre-moistening DS in skim milk (Byrd et al., 1997). Culturing olefin-spun boot covers **(BC)** worn over plastic boot covers in poultry houses was shown to be as effective as DS sampling (Caldwell et al., 1998) and using dry elastic cotton tube socks were as effective as collecting fecal samples by hand (Skov et al., 1999). In more recent studies, wearing melt-blown polypropylene dry surgical socks or olefin-spun BC similar to those utilized by Caldwell et al. (1998) showed a higher sensitivity than DS (McCrea et al., 2005; Mueller-Doblies et al., 2009), and the use of boot socks soaked in saline showed higher detection than DS, LG, and fecal samples (Buhr et al., 2007). Sensitivity of the different litter sampling methods seems to vary based on litter conditions, level of *Salmonella* prevalence, and preparation of the swab media. The objective of this study was to employ the use of Roller Swabs **(RS)** in litter sampling and to compare it to LG, DS, and BC culture methods following two sequential flocks of broilers. Two experiments were performed; in the first experiment litter was sampled for a two-week period after oral challenging a whole flock with SE in order to establish a high prevalence of S in the litter and in the second experiment litter was sampled following a flock raised on the same litter as the first flock to sample when the prevalence of S was lower, environmental challenge.

MATERIALS AND METHODS

Experiment 1 Design

Commercially obtained broiler chicks were spread over 32 separate 1.52 m^2 pens (25 birds/pen) onto fresh pine shavings. Pens were arranged into four rows of eight pens each with two adjacent rows on each side of the house.

All chicks were challenged with 1 ml of a suspension of 10^7 cfu/ml of a nalidixic acid and novobiocin resistant strain of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) at 6 days of age by oral gavage using 1 cc tuberculin syringes. The isolate was prepared from beads that were stored at -80°C and then transferred onto tryptic soy agar with 5% sheep's blood (Catalog #10128-598, VWR Scientific, Radnor, PA) and incubated at 37°C for 18-24 hours. The SE isolate was confirmed as *Salmonella* Group D using Difco Salmonella O Antiserum Poly A- I & Vi (Catalog # 222641, Becton, Dickinson, and Company, Sparks, MD). One colony was then streaked onto Xylose Lysine Tergitol 4 (Catalog #C8032 Criterion Dehydrated Culture Media, Hardy Diagnostics, Santa Maria, CA) containing 100 µg/ml of nalidixic acid (Catalog # J63550-06 Alfa Aesar, Haverhill, MA) and 15 µg/ml of novobiocin (Catalog #J60928-09, Alfa Aesar) (XLT4+) and incubated for 18-24 hours at 37°C. SE inoculum was prepared via overnight culture by inoculating Brain Heart Infusion Broth (BHIB, Catalog #C5141 Hardy Diagnostics) with one colony of SE that was isolated on XLT4+ and then placed into a New Brunswick Innova 4300 Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for 18-24 h with 200 revolutions per minute (RPM) at 37°C. BHIB overnight culture was then diluted 100x with 1x Phosphate Buffered Saline (PBS) (Catalog #75800-998, VWR Scientific) to prepare the proper challenge dose of $1x10^7$ cfu/ml. BHIB overnight culture

was enumerated by making dilutions to spread plate onto XLT4+ and was determined to contain $1.72x10^9$ cfu/ml of SE.

The birds were fed a standard starter, grower, and finisher pelleted diet consisting of soybean and corn meal. The flock was terminated at 35 days. Ceca were collected from 160 birds (5 birds per pen) on day 35. All litter sampling methods were performed in 5 separate sampling periods. The first sampling occurred on the day of termination (D0) after bird removal, four days later (D4), seven days post termination (D7), ten days post termination (D10), and the final sampling occurred at thirteen days post termination (D13).

Experiment 2 Design

Commercially obtained broiler chicks were spread over the same 32 pens used in the first experiment. This flock of birds was reared using the same feed formulations and management scheme as the initial experiment. This second flock was reared to 45 days of age. At day 42, ceca were collected from 160 birds (5 birds per pen), and the remaining birds were terminated on day 45. Litter sampling was performed one week before ceca collection (D-10 bird age 35 days), on the day of ceca collection (D-3), 4 days after termination of the flock (D4), and one week after termination of the flock (D7).

Sampling Procedure

Five birds per pen were randomly selected to collect ceca. Each bird was euthanized, and ceca were collected aseptically, sliced several times down the length and ends of ceca, and placed into 118 ml puncture proof bags (Catalog #11216-012, VWR, Nasco Whirl-Pak, Madison, WI) and set on ice prior to transport to the lab. Ceca were then enriched in Tetrathionate Broth (TT, Catalog #C7062, Hardy Diagnostics) with 2% Iodine-Iodide solution at 37°C for two

days prior to mixing the sample by massaging the bag by hand and streaking onto XLT4+ into quadrants with 1 µl disposable plastic inoculation loops (Catalog #89126-872, VWR Scientific). Litter grab composite samples for each pen were created from three areas of each pen. From each area, ~50 g of litter was collected; these areas were beside the feeder, under the water lines, and in between the two and placing the collected litter into 532 ml puncture proof bags (Catalog #11216-056, VWR, Nasco Whirl-Pak) (Figure 3.1). Litter samples were enriched by adding 1 g of litter to 9 ml of TT broth with 2% Iodine-Iodide solution into 50 ml conical vials (Catalog # 89039-658, VWR Scientific) and incubated at 37°C for 48 ± 2 h. After that time 10 µl was streaked on XLT4+ in quadrants with 10 µl disposable plastic inoculation loops (VWR Scientific).

Drag swabs were made in the laboratory by cutting 15 cm by 15 cm squares of cheese cloth (Catalog #100488-116, VWR, Electron Microscopy Sciences, Hatfield, Pennsylvania) and tying to 1 m of twisted mason line nylon string (Item #38LY41, Model #BC347, W.W. Grainger Inc., Lake Forest, IL) (Figure 3.2). The assembled DS were placed into 50 ml conical vials (VWR Scientific) and presoaked in 10 ml of double strength skim milk prepared from nonfat powdered milk (#9278117, Walmart Inc., Bentonville, AR) and then autoclaved at 121°C for 15 min. Sampling was performed by aseptically removing the DS from the conical vial with a clean pair of gloves and dragging the swab over the surface of the litter in each pen vertically and horizontally, however due to equipment in the pens this was approximately 75% of the pens surface area. Afterwards, the DS was placed back into the vial and gloves were changed before sampling each pen. The DS media was enriched by adding 30ml of TT broth with Iodine-Iodide solution to the conical vial and incubating at 37°C for 48 ± 2 h. After incubation, the vial was vortexed and the sample was streaked onto XLT4+ in quadrants with 10 µl loops.

Boot cover sampling was performed in each pen using Hardy Diagnostic Enviro Bootie boot covers (Catalog # EB100, Hardy Diagnostics, $422/100) that were presoaked in skim milk and placed into their original, sterile bag. Sampling procedure involved aseptically placing the BC over a fresh plastic disposable boot cover and then walking inside the pen to cover approximately 75% of the surface (Figure 3.3). The BC was then removed aseptically and placed back into its original packaging. Gloves and plastic boot covers were removed and replaced after each pen to prevent cross contamination. The BC was enriched by adding 50 ml of TT broth with 2% Iodine-Iodide solution to the bag, massaged by hand for 15 seconds, and then incubating at 37°C for 48 ± 2 h. After incubation, the BC bag was massaged by hand and the media was streaked onto XLT4+ in quadrants with 10 µl loops.

RS sampling media was prepared by using 10.16 cm x 1.27 cm paint roller covers (Item #6LFG8, Model #84072, W.W. Grainger Inc.; $130/100) and aseptically placing them into a 50 ml conical vial containing 20 ml of double skim milk. Sampling was performed in each pen by using a standard paint roller handle to remove the RS from the vial (Figure 3.4). The RS was then rolled over approximately 75% of the surface area of the pen, while also applying downward pressure. Rollers were then aseptically placed into 120 ml puncture proof bags (Nasco Whirl-Pak). The RS were enriched by placing 50 ml of TT broth with 2% Iodine-Iodide solution into the bags, massaging by hand for 15 s, and then incubating at 37°C for 48 ± 2 h. After incubation, the RS bag was massaged by hand for 15 s and then streaked in quadrants onto XLT4+ with 10 µl loops.

Statistical Analysis

All statistical analyses were conducted using SPSS Software version 26 (IBM, Armonk, NY). The number of positive samples between sampling methods, between sampling days within each sampling method, and between ceca enrichment were analyzed using chi-squared test. Significant differences were reported at $P \leq 0.05$.

Animal Ethics

These experiments were conducted in accordance with the Institutional Animal Care and Use Committee at Auburn University.

RESULTS AND DISCUSSION

Experiment 1

In experiment 1, 98% (157/160) of LG, 97% (156/160) of BC, 95% (152/160) of DS and 81% (129/160) RS were found positive for S (Table 1). It was observed that RS S detection was significantly lower than the other methods tested ($P < 0.0001$). Although S detection using RS did not differ from the other methods at D0 and D4, detection using RS decreased significantly after D4 (32/32) to D7 and D13 (24/32) ($P = 0.0048$) and further on D10 (17/32) ($P < 0.01$) (Table 3.1). BC, DS, and LG sampling methods did not change over time. Ceca collected from the flock on D0 had 34% (54/160) positive samples (Table 3.2), which demonstrated a high prevalence of S in the flock.

No overall differences were observed between common litter sampling methods BC, DS, and LG in Experiment 1 when *Salmonella* was highly prevalent, which agreed with similar studies with challenged birds and pens having a high prevalence of *Salmonella* (Buhr et al., 2007). The RS method was less sensitive compared to the other methods, possibly due to the less litter adherence to the RS surface. Although applying pressure onto the surface of litter with the RS could help with adherence, it was noticed that not as much litter or feces clung to the RS in

comparison to DS or BC. Detection of *Salmonella* with RS decreased over time, although no other tests indicated a reduction in S prevalence. Concerning ease and practicality of use in a research pen environment, the RS were easier to use than DS, BC, and LG sampling and preparation was less labor intensive than preparing DS. In this study, the RS were easier to use than the DS, BC, and LG due to the need to change plastic boot covers aseptically in between pens for research trials, however, this would not be the case if sampling a commercial broiler house.

Experiment 2

In Experiment 2, 55% (71/128) of BC, 23% (29/128) of RS, 17% (22/128) of LG, and 6% (8/128) of DS were positive for S (Table 3.3). Differences in S detection were between BC and all other methods ($P < 0.001$), between DS and RS ($P < 0.001$), and between LG and DS ($P < 0.01$) (Table 3.3). The recovery of S from the ceca of the flock from Experiment 2 was 3% (4/160) (Table 3.4) which was significantly lower than the first flock ($P < 0.0001$). Differences were observed between D-10 and all other sampling days with D7 in the RS group with 47% (15/32) positive samples on D-10 decreasing to 19% (6/32) on D-3 and D4 ($P < 0.05$) and 9% (2/32) on D7 ($P < 0.001$) although statistically significant reduction over time was not observed in other sampling methods.

Recovery of *Salmonella* in Experiment 2 was higher for the BC method compared to all other methods in conditions with a lower prevalence of *Salmonella*, which has been observed in studies using similar methods (McCrea et al., 2005; Buhr et al., 2007; Mueller-Doblies et al., 2009). The use of RS was more effective than DS in recovering S, which may be attributed to the pressure applied with RS onto the litter. The pressure applied to the litter surface improves litter sampling for *Salmonella* detection which is demonstrated by the increased frequency of

Salmonella detection of similar BC sampling methods in previous studies (McCrea et al., 2005; Buhr et al., 2007; Mueller-Doblies et al., 2009), and the concept has been further demonstrated by stepping on DS to increase *Salmonella* detection (Buhr et al., 2007). In addition to the ease of use observed in Experiment 1, it was noted that broilers were more likely to move out of the way for the RS. In contrast, broilers appeared less likely to move for the DS and attempted to peck at the DS during sampling. Additionally, during BC sampling, birds tended to bunch into the pen's corners. The RS method has advantages over other sampling methods when used for research pens, as it was easy to use and can be used to sample litter without entering the research pen. Therefore, the RS method can be a potential method for detection for *Salmonella* from poultry litter in a research setting while the BS method is the most sensitive method and is also the most convenient for sampling conventional poultry houses.

CONCLUSIONS AND APPLICATIONS

- With high *Salmonella* environmental prevalence, Roller Swab sampling was less sensitive for *Salmonella* detection than Boot Swabs, Drag Swabs, and Litter Grab techniques.
- With lower *Salmonella* environmental prevalence, Boot Covers were the most sensitive method for *Salmonella* detection, while Roller Swabs and Litter Grab were more sensitive than Drag Swabs in these environments.
- Roller Swabs may be more practical to use than Drag Swabs in sampling research pens. They may also be more practical to use than Boot Covers and Litter Grab when sampling individual research pens and not necessitate the purchase of plastic boot covers for each pen to be sampled.

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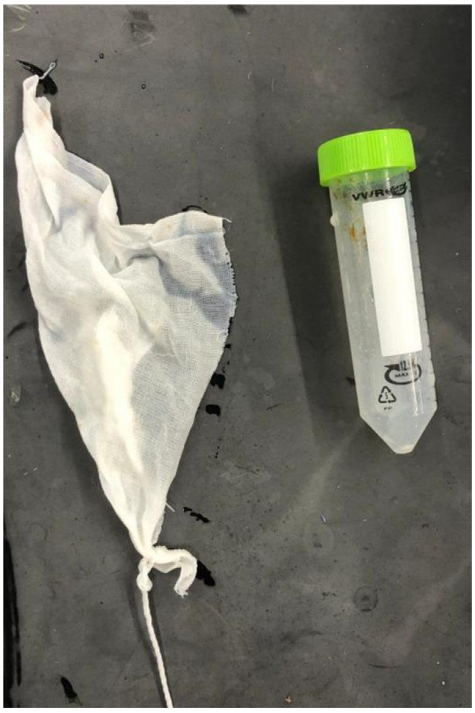
FIGURES

Figure 3.1: Photograph of the Litter Grab method

Description: This picture depicts litter grab sampling by taking handfuls of litter with a fresh glove and placing into a sterile puncture-proof bag.

Figure 3.2: Photograph of the Drag Swabs Assembled in the Laboratory



Description: This figure depicts the drag swabs assembled in the lab by cheese cloth and string and the tube the swab was sterilized in prior to sampling.

Figure 3.3: Photograph of the Boot Cover Method

Description: This figure depicts boot cover sampling by aseptically placing a boot cover over a fresh plastic boot cover and stepping on the surface of the litter.

Figure 3.4: Photograph of the Roller Swab attached to the roller handle for sampling

Description: This figure shows the paint roller attached to the roller handle for sampling and the tube utilized for storage of the roller swab prior to sampling.

DATA

Table 3.1: Experiment 1; Comparison of *Salmonella* Enteritidis recovery between litter sampling methods following a broiler flock with high prevalence of *Salmonella*

Day	Litter Grab	Drag Swab	Boot Cover	Roller Swab
D0	31/32	32/32	32/32	32/32
D4	32/32	32/32	31/32	32/32
D7	32/32	29/32	29/32	24/32*
D10	31/32	30/32	32/32	17/32*
D13	31/32	29/32	32/32	24/32*
Total	157/160	152/160	156/160	129/160
%	98[a]	95[a]	97[a]	81[b]

[a-b] Values within a row with differing letters are significantly different ($P \leq 0.05$).

* Values within a column with differing symbols are significantly different ($P \leq 0.05$).

Table 3.2: Experiment 1; Comparison of *Salmonella* recovery from ceca between pens

Pen #	Positive Samples	Pen #	Positive Samples
1	1/5	17	2/5
2	0/5	18	2/5
3	1/5	19	3/5
4	3/5	20	3/5
5	2/5	21	2/5
6	1/5	22	4/5
7	0/5	23	1/5
8	2/5	24	4/5
9	0/5	25	1/5
10	1/5	26	3/5
11	0/5	27	5/5
12	1/5	28	0/5
13	2/5	29	2/5
14	1/5	30	2/5
15	1/5	31	1/5
16	2/5	32	1/5

Table 3.3: Experiment 2; Comparison of *Salmonella* recovery between litter sampling methods following a broiler flock with low prevalence of *Salmonella*

Day	Litter Grab	Drag Swab	Boot Cover	Roller Swab
D-10	2/32	5/32	18/32	15/32
D-3	6/32	2/32	18/32	6/32*
D4	6/32	1/32	17/32	6/32*
D7	8/32	0/32	18/32	2/32*
Total	22/128	8/128	71/128	29/128
%	17[b]	6[c]	55[a]	23[b]

[a-b] Values within a row with differing letters are significantly different ($P \leq 0.05$).

* Values within a column with differing symbols are significantly different ($P \leq 0.05$).

Table 3.4: Experiment 2; Comparison of *Salmonella* recovery from the ceca between pens

Pen #	Positive Samples	Pen #	Positive Samples
1	0/5	17	1/5
2	0/5	18	0/5
3	0/5	19	0/5
4	0/5	20	0/5
5	0/5	21	0/5
6	0/5	22	0/5
7	0/5	23	1/5
8	0/5	24	0/5
9	0/5	25	0/5
10	0/5	26	0/5
11	0/5	27	0/5
12	0/5	28	0/5
13	0/5	29	0/5
14	1/5	30	0/5
15	0/5	31	0/5
16	1/5	32	0/5

Chapter 4:

Examining the Effects of Litter Treatments on the Persistence of *Salmonella* Enteritidis in Poultry Litter

By A.A. Talorico

SUMMARY

Nontyphoidal *Salmonella* contributes to a significant number of cases of foodborne illness in the United States. Reuse of litter in broiler houses can lead to the accumulation of *Salmonella* spp. in litter. The purpose of this study was to evaluate the effect of *Bacillus* spp. Probiotic blend (PB), Sodium Bisulfate (SB), Sodium Formate Salts (SF), and Windrow Composting (WC) treatments on the persistence of *Salmonella* Enteritidis in poultry litter after challenging one flock with 10^7 cfu of nalidixic acid resistant *Salmonella* Enteritidis (SE) and raising three flocks on the same litter. One thousand commercially sourced chicks were spread over 40 pens (25/pen) with 8 pens per treatment and were challenged with 10^7cfu of a nalidixic acid resistant strain of *Salmonella* Enteritidis (SE) at 6 days of age. Composting was performed by removing litter from all WC pens and creating a pile for 6 days during downtime and other treatments were applied the day before chick placement. SB was applied at 100lb/1000ft^2, PB at 10lb/1000ft^2, and SF at 40gallons/1000ft^2. SE prevalence was assessed during the downtime between flocks. Boot Covers (BC) and Litter Grab (LG) were pre-enriched in Tetrathionate broth and streaked on selective media. Results were assessed based positive or negative results and analyzed by chi-square test. Additionally, LG were enumerated for SE and analyzed by GLM. Sampling performed during the downtime after the rearing of the seeder flock will be referred to as Experiment 1 (E1) and the subsequent flocks referred to as Experiments 2, 3, and 4 (E2, E3, and E4). There were no differences in SE prevalence in either LG or BC in E1. No differences were observed in LG SE counts between groups in any experiment. However, differences in E2 were observed in LG, with SB being greater than UC, WC, and SF and PB having greater prevalence than SF ($P<0.046$). No differences were present between groups in BC during any of the sampling periods. Additionally, the ceca of market age birds were collected in to evaluate if any treatments affected the transmission of SE from the seeder flock to the subsequent flocks. No

differences were observed in cecal colonization of SE between groups. This data suggests that SE persistence and transmission between flocks was not affected by any litter treatment.

DESCRIPTION OF PROBLEM

Nontyphoidal *Salmonella* remains one of the top foodborne illnesses in hospitalizations and deaths associated with infection (CDC, 2019). *Salmonella* Enteritidis **(SE)** is a frequently isolated serotype of *Salmonella* spp. in poultry production, with high pathogenicity in humans (CDC, 2016). Contamination of poultry litter can lead to the spread of *Salmonella* spp. within a flock and to subsequent flocks that are raised on the same litter. Broilers become infected with *Salmonella* spp. by intraoral or cloacal uptake of contaminated litter or by aerosolized *Salmonella* spp. in dust. This model was established by challenging birds orally, intratracheally, or by aerosolizing contaminated poultry dust and then recovering *Salmonella* from the ceca of birds (Barrow, 1991; Nakamura et al., 1995; Gast et al., 1998; Bailey et al., 2006; Chadwick et al., 2020, Pal et al., 2021), which is the most readily infected organ in broilers (Fanelli et al., 1971; Snoeyenbos et al., 1982). *Salmonella* spp. is known to persist in litter for months to years due to its resilience and adaptability in the environment (Davies & Wray, 1996), which allows for transmission between flocks raised on the same litter. Litter sampling has been utilized as a practical and non-invasive method to determine flock Salmonella status. The use of boot covers (**BC**) as a sampling method for *Salmonella* spp. detection was first reported in 1998 by Caldwell et al. and has been observed to be the most sensitive method for *Salmonella* spp. detection by multiple groups in research and industrial settings (Caldwell et al., 1998; Skov et al., 1999; McCrea et al., 2005; Buhr et al., 2007; Talorico et al., 2021). Litter sampling by forming a composite sample of poultry house litter, known as litter grab sampling (**LG**), has been used for the detection and enumeration of *Salmonella* spp., anaerobic bacteria, aerobic bacteria, and other

pathogens (McCrea et al., 2008; Z. Williams et al., 2012). In addition to the detection of bacteria, LG can be used to determine litter moisture or litter water activity which may be an indicator for aerobic bacteria, enteric bacteria, or *Salmonella* spp. survivability (Turnbull & Snoeyenbos, 1973; Carr et al., 1995; Payne et al., 2007)

In the United States it is a common practice to reuse litter in between broiler flocks, and the reuse of litter has been observed to have some inhibitory effect on the spread of *Salmonella* spp. versus the use of new litter (Olesiuk et al., 1971). Additionally, it has been reported that *Salmonella* spp. prevalence decreases over time as litter is reused (Roll et al., 2011). However, litter treatment and management practices are necessary to reduce litter ammonia buildup which can reduce bird performance and is a concern for flock welfare (Miles et al., 2004; Reece et al., 1980). Acidifying litter with Sodium Bisulfate ($NaHSO_4$) (**SB**) or Aluminum Sulfate ($Al_2(SO_4)_3$) has been observed to reduce ammonia levels in built up litter, but no reduction in *Salmonella* spp. has been reported when acidifying the litter with these treatments (Burgess et al., 1998; Pope & Cherry, 2000; Williams et al., 2012). Pope and Cherry (2000) reported a reduction in total bacterial and *Escherichia coli* counts in litter with the use of SB. Williams et al. (2012) observed that the use of SB at 45.4 kg/92.9m^2 can decrease litter pH to nearly neutral, which can benefit the survivability of *Salmonella* spp. and other enteric bacteria; nonetheless, Payne et al. (2002) saw a reduction in *Salmonella* spp. at a similar rate of application with a greater decrease in litter pH. However, these differences are most likely due to differences in methodology with one group sampling litter from under the surface of litter versus sampling only from the top layer of litter (Payne et al., 2002; Williams et al., 2012). Although acidifying the litter with aluminum sulfate or SB does not appear to have an effect in reducing *Salmonella* spp. prevalence in poultry litter, some projects have observed the use of granular sulfuric acid (H_2SO_4) to be effective in

reducing or eliminating *Salmonella* spp. in litter in addition to reducing ammonia levels (Payne et al., 2002; Williams & Macklin, 2013).

An additional measure that poultry farmers can use to improve litter quality is in-house windrow composting (**WC**), which is performed by pushing litter into long rectangular piles in broiler houses. This method has been reported to reduce litter moisture content while also reducing aerobic, anaerobic, coliform, *Salmonella* spp., *Clostridium perfringens*, and *Campylobacter* counts (Macklin et al., 2006, 2008). The complete pasteurization of reused litter is accomplished by maintaining core temperatures greater than 55℃ for 3 days, which has a pasteurization effect against bacteria (Jones & Martin, 2003). The thermal death point of *Salmonella* spp. in manure was reported to be 55℃ for 1 hour or 60℃ for 20 minutes by Jones and Martin in 2003. Turning the piles after a few days can reintroduce oxygen to microbes breaking down organic materials and can help achieve homogenous pasteurization (Macklin et al., 2006). This method is effective in maintaining litter quality, is low-cost, and is less labor intensive than removing litter.

The use of probiotics with *Bacillus* species has been investigated in reducing *Salmonella* spp. contamination of the ceca and litter of broilers. Administration of *Bacillus* spores in feed has been observed to reduce *Salmonella* spp. colonization of the ceca, shedding of *Salmonella* in the feces, and a decrease of *Salmonella* spp. contamination of the carcass of broilers (Fritts et al., 2000; Knap et al., 2011; La Ragione & Woodward, 2003; Vilà et al., 2009) in addition to increasing bird weight and feed conversion in some studies (Chiang & Hsieh, 1995; Fritts et al., 2000; Lee et al., 2014). However, employing *Bacillus* probiotics blends (**PB**) as a litter treatment is less investigated, although De Cesare et al. (2019) observed a decrease in total aerobic and *Enterobacteriaceae* counts after using PB as a litter treatment, and Pezzuolo et al. (2019)

observed a reduction in ammonia emissions after using PB as a litter treatment. Further research is needed to verify if the application of PB as a litter amendment is effective in the reduction of bacterial load or *Salmonella* spp. levels in reused litter.

Sodium Formate salts (**SF**) are a liquid byproduct of plastic production containing a mixture of short chain acids, but primarily the salts of formic acid. Sodium formate has common uses as a fabric dying agent and as a deicer for airport runways, however, the mixture referred to as Sodium formate salts likely has major differences than pure sodium formate solution. The efficacy of SF as an antimicrobial has not been evaluated in published research, however, SF theoretically has antimicrobial capabilities.

The first objective of this study was to observe the persistence and spread of *Salmonella* spp. in subsequent broiler flocks after challenging all birds of the first flock to seed the litter. The second objective was to observe the effect of five litter treatments on persistence and spread while raising subsequent naïve flocks on the same litter. The five treatments included: an untreated control (**UC**), *Bacillus* probiotic blend (**PB**), Sodium Bisulfate (**SB**), Windrow Composting (**WC**), and Sodium Formate salts (**SF**).

MATERIALS AND METHODS

Experiment 1 Design

For Experiment 1 (E1), 1000 commercially obtained straight run broiler chicks were spread over 40 separate 1.52 m^2 pens (25 birds/pen) on fresh pine shavings. Pens were arranged into four rows of ten pens each with two adjacent rows on each side of the house. The birds were fed a standard starter, grower, and finisher diet that met or exceeded the NRC suggested minimum nutrient requirements of broilers (NRC, 1994).

All chicks were challenged with 1 ml of a suspension of 10^7 cfu/ml of a nalidixic acid and novobiocin resistant strain of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) at 6 days of age by oral gavage using 1 cc tuberculin syringes. The isolate was prepared from beads that were stored at -80°C and then transferred onto tryptic soy agar with 5% sheep's blood (VWR Scientific, Radnor, PA) and incubated at 37°C for 18-24 hours. The SE isolate was confirmed as *Salmonella* using Difco Salmonella O Antiserum Poly A- I & Vi (Becton Dickinson, Sparks, MD). One colony was then streaked onto Xylose Lysine Tergitol 4 (Hardy Diagnostics, Santa Maria, CA) containing 100 µg/ml of nalidixic acid (Alfa Aesar, Haverhill, MA) and 15 µg/ml of novobiocin (Alfa Aesar) (XLT4+) and incubated for 18±1 hours at 37°C. SE inoculum was prepared via overnight culture by inoculating 50ml of Brain Heart Infusion Broth (BHIB; Hardy Diagnostics) with one colony of SE that was isolated on XLT4+ and then placed into a New Brunswick Innova 4300 Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for 18-24 h with 200 revolutions per minute (RPM) at 37°C. BHIB overnight culture was then diluted 100x with 1x Phosphate Buffered Saline (PBS; VWR Scientific) to prepare the proper challenge dose of $1x10^7$ cfu/ml. BHIB overnight culture was enumerated by making serial dilutions and spread plating onto XLT4+. The challenge dose of SE was determined to be $1.72x10^9$ cfu/ml.

The flock for E1 was terminated at 35 days, and ceca were collected from 200 birds (5 bird/pen) at this time. All litter sampling methods were performed in 5 separate sampling periods. The first sampling occurred on the day of termination (D0), then four days later (D4), seven days (D7), ten days (D10), and at thirteen days (D13) post termination.

The WC treatment was performed by collecting all litter from WC pens after sampling on D4 and placing it into two separate 2 m x1 m x 1 m piles, each pile consisted of litter from four pens within adjacent rows. Windrow piles were broken up after one week and placed back into the pens corresponding to the two rows the litter was collected from prior to sampling on D10. Windrow piles on D7 were sampled by performing litter grab samples by collecting a composite sample from the surface and from 1 m in depth. The PB, SB, and SF treatments were applied approximately 3 hours prior to sampling on D13, which was within 24 hours of placement of chicks, as outlined by the manufacturer's instructions. PB was applied at 0.45 kg/92.90 m^2 , SB was applied to pens at 45.35 kg/92.90 m^2, SF was applied at 151.4 L/92.90 m^2.

Experiment 2 Design

For experiment 2 (E2), 1000 commercially obtained straight run broiler chicks were spread over the same 40 pens used in the first experiment. This flock of birds was reared using the same feed formulations and management scheme as E1. This second flock was reared to 45 days of age. At day 42, ceca were collected from 200 birds (5 birds per pen), and the remaining birds were terminated on day 45. Litter sampling was performed one week before ceca collection (D-10), on the day of ceca collection (D-3), 4 days after termination of the flock (D4), and one week after termination of the flock (D7).

For E2, the WC pile was created after sampling on D0 and was broken up and placed back into corresponding pens prior to sampling on D7. For this trial, one single windrow pile was formed, and the internal temperature of the pile was monitored using two USB temperature data loggers (Omega Engineering Inc., Czech Republic). All litter cake was broken up prior to the application of treatments. The PB, SB, and SF treatments were applied three hours prior to

sampling on D7, which is the day before placement of chicks from the subsequent flock. All treatments were applied in the same dosages as were used in E1.

Experiment 3 Design

For Experiment 3 (E3), 1000 commercially obtained straight run broiler chicks were spread over the same 40 pens used in E1 and E2. This flock of birds was reared using the same feed formulations and management scheme as the initial experiment. This flock was reared to 45 days of age. At day 42, ceca were collected from 200 birds (5 birds per pen), and the remaining birds were terminated on day 45. Litter sampling was performed one week before ceca collection (D-10 bird age 35 days), on the day of ceca collection (D-3), 4 days after termination of the flock (D4), and one week after termination of the flock (D7), and 11 days after termination of the flock (D11). After sampling on D11, litter samplings was performed weekly until the placement of the next flock (D103). Nalidixic acid susceptible *Salmonella* (NS) was detected during this sampling schedule and was monitored from this point on using XLT4 without antibiotics. Due to the inability to obtain chicks at hatcheries because of COVID-19 restrictions, chicks were unobtainable for a 15-week downtime, so SE and NS was monitored weekly until placement of the next flock (D104). One windrow compost pile was formed after sampling on D0 and was broken up on D11 prior to sampling, although the remaining treatments were not applied until D103, which was the day before chick placement for Experiment 4. To monitor temperature, one USB data logger was placed at the bottom of the windrow pile (1 m deep), at 0.5 m in depth, and on the surface of the pile.

Experiment 4 Design

For Experiment 4 (E4), 1200 commercially sourced straight run chicks were placed in 48 pens (25 chicks/pen) with chicks placed in the same 40 pens as the prior experiments with an additional 2 pens added on the end of each of the four rows. These 8 additional pens contained fresh bedding and was used for a sixth treatment group – fresh bedding (FB). This flock was reared in the same manner as in previous experiments (E1-3) and was terminated and had ceca collected on Day 42. In this experiment, collected LG samples were enumerated for total aerobic, total coliform, and *E. coli* counts.

Sampling Procedure

Five birds per pen were randomly selected to collect ceca. Each bird was euthanized, ceca collected aseptically, sliced, placed into 118 ml puncture resistant bags (VWR, Nasco Whirl-Pak, Madison, WI) and set on ice prior to transport to the lab. Ceca were then enriched in Tetrathionate Broth (TT, Hardy Diagnostics) with 2% Iodine-Iodide solution at 37°C for two days prior to mixing the sample and streaking onto XLT4+ into quadrants with 10µl disposable plastic inoculation loops (VWR Scientific). For Experiments 3 and 4, a pre-enrichment step was utilized by adding 25 ml of 2% Buffered Peptone Water (BPW; VWR Scientific) to the sampling bag and then incubated at 37°C for 24h. After incubation, the pre-enriched ceca bag was massaged by hand for 15 s and 1 ml of the pre-enrichment media was added to 9 ml of TT in 15ml conical vials. After incubation at 37°C for 48 ± 2h, the conical vials were vortexed and the media was streaked onto XLT4+ in quadrants with 10 µl loops.

LG samples for each pen were created from three areas. From each area, ~50 g of litter was collected; these areas were beside the feeder, under the water lines, and in between the two. Collected litter was placed into 532ml puncture resistant bags (VWR, Nasco Whirl-Pak). Litter samples were enriched by adding 1g of litter into 15ml conical vials (VWR Scientific) containing

9 ml of TT broth with 2% Iodine-Iodide. The vials were then incubated at 37°C for 48 ± 2h. After incubation, 10 µl of TT enrichment was streaked on XLT4+ in quadrants with 10µl disposable plastic inoculation loops (VWR Scientific).

LG samples were enumerated by placing 10g of litter into Whirl-Pak Homogenizer Filter Bags (Nasco Whirl-Pak, Madison, Wisconsin), diluting with 90 ml of PBS (VWR Scientific), and stomaching in an easyMIX® Lab Blender (AES-Chemunex, France) for one minute each. Each sample was enumerated on XLT4+ by spot plating 10µl onto grid plates (VWR Scientific) in triplicate and then incubating the plates at 37°C for 48±2 hours before counting colonies. Colonies were identified as *Salmonella* by morphology and black colony color, between 1 and 25 colonies were counted per spot. In E4 LG samples were enumerated on Plate Count Agar (PCA; VWR Scientific) and MacConkey Agar (VWR Scientific) by diluting 10g of litter with 90ml of PBS and placing dilutions of 100 ul onto each plate in triplicate and spread plating. All plates were then incubated at 37°C. Total aerobic counts were assessed based on the total number of colonies (30-300) on PCA. *E. coli* counts were observed by counting colonies (30-300) on MacConkey medium that were pink in color, indicating lactose fermentation, and with morphology characteristics of *E. coli*. Total coliform counts were observed by counting all colonies on MacConkey agar. An average count between the three replicates in each sample was used to determine colony forming units (cfu).

Samples to obtain litter moisture were performed by weighing 10 g of litter in tin dishes (VWR) and then placing the dishes into a convection oven (Sheldon Manufacturing Inc., Cornelius, Oregon) at 90°C for 48±2 hours and then measuring the final weight. Litter moisture percentage was determined by dividing the difference between the initial and final weight by 10 and then multiplying the decimal by 100.

Boot cover sampling was performed in each pen using Hardy Diagnostic Enviro Bootie boot covers (Hardy Diagnostics) that were presoaked in skim milk and placed into their original, sterile bag. Sampling involved aseptically placing the BC over a clean plastic disposable boot cover and then walking inside the pen to cover approximately 75% of the surface. The BC was then aseptically removed and placed back into its original packaging. Gloves and plastic boot covers were removed after each pen to prevent cross contamination. The BC was enriched by adding 50 ml of TT broth with 2% Iodine-Iodide solution to the bag, massaging by hand for 15s, and then incubating at 37°C for 48 ± 2 h. After incubation, the BC bag was massaged and the media streaked onto XLT4+ in quadrants with 10 µl loops. For E3 and E4, a 18-24h pre-enrichment step was utilized. The pre-enrichment consisted of adding 50 ml of 2% BPW to the sampling bag and then incubated at 37°C for 24 h. After incubation, the BC bag was massaged for 15s and 10ml of the pre-enrichment media was added to 35 ml of TT in 50 ml conical vials. After incubation at 37°C for 48±2h, the conical vials were vortexed and the media was streaked onto XLT4+ in quadrants with 10 µl loops.

Statistical Analysis

All statistical analyses were conducted using SPSS Software version 26 (IBM, Armonk, NY). The number of positive samples between litter treatment groups; also between sampling days within each treatment group, and ceca enrichments were analyzed using chi-squared test or Fisher's exact test if appropriate. All average bacterial counts were log transformed and litter moisture samples were arcsine transformed and then analyzed by Generalized Linear Model if significant means were separated by Tukey's HSD. Significant differences were reported at $P \leq 0.05$.

RESULTS AND DISCUSSION

Experiment 1

Table 4.1 summarizes the recovery of *Salmonella* from the ceca on the day of flock termination for E1 (D0) and compares pens designated for each treatment group before treatments were applied. Ceca collected from birds in WC pens were found to be 52% positive which was similar to 45% in the SF pens and 32% in the PB pens, but greater than 30% in the SB pens (P=0.04) and 27% in the UC pens (P=0.02). The enrichment of ceca for SE recovery during E1 primarily served to verify challenge uniformity between treatment groups before treatments were applied, although differences were observed between birds sampled from different pens (Table 4.1). These differences between groups are most likely due to the change in the gut microflora of birds over time because all birds were challenged at six days of age. Additionally, only a sample of the population was taken (5/pen) and the differences may be due to chance.

Salmonella counts within the WC group were analyzed to observe the changes in the *Salmonella* load before and after the formation of the compost piles by (Figure 4.1). No statistical differences were observed between D0, D4, D7, D10, or D13 ($P > 0.05$). Larger decreases in SE counts could be possible with turning the pile and/or allowing a longer time for pasteurization. Complete pasteurization of composted litter was achieved by Macklin et al. in 2008 without turning the pile, although non-composted samples still retained some *Salmonella* spp. (Macklin et al., 2008). The differences between the findings of this experiment and the findings of Macklin et al. (2008) could be due to differences in methodology. This experiment seeded the litter with SE by challenging a whole flock versus inoculating samples and placing them within the compost piles. The lack of turning in this experiment did not allow the entire pile to be exposed to temperatures that could kill SE. This further demonstrates the necessity of

turning Windrow Compost piles for the complete killing of *Salmonella* spp. from litter, especially in litter that is seeded with *Salmonella*.

The detection of *Salmonella* by BC sampling from the day of flock termination (D0) to the day before placement of the preceding flock (D13) is summarized in Table 4.2. No differences were observed between sampling days or treatments due to nearly all samples being positive for SE . The treatments that were applied before sampling on D13 did not affect prevalence of SE and the WC treatment applied after sampling on D4 and before sampling on D10 also had no effect on SE prevalence.

The comparison of LG *Salmonella* counts between treatment groups on D10 and D13 is displayed by Figure 4.2. Although no statistical differences were observed between groups on D10 or D13 or within groups between D10 and D13, a numerical increase in counts from D10 to D13 was observed in UC, PB, and WC groups. Although no statistical differences were observed, a .84log decrease was observed in the SF group between D10 and D13 while increases in SE counts were observed in UC, PB, and WC. The SB group did decrease SE counts by .37 logs which is in agreeance with previous literature about SB having no immediate effect on *Salmonella* spp. prevalence (Williams et al., 2012; Williams & Macklin, 2013). There was a small numerical increase in counts in groups which may have been due to heating the house for the placement of chicks of the next flock. This data suggests that there is no immediate effect on SE prevalence with the applications of these treatments on D13.

Experiment 2

The recovery of *Salmonella* from the ceca of birds terminated on D-3 of E2 is shown in Table 4.3. No statistical differences were observed between groups with 0% recovery in UC and SF groups, 2% in WC and SB groups, and 7% in the PB group. It could be expected that litter

with a higher *Salmonella* prevalence could be a greater risk for *Salmonella* colonization of the GI tract in chicks and then spread *Salmonella* further throughout the flock, but there were no differences in SE counts between groups on D13 of E1 (Figure 4.2). The lack of difference in SE prevalence between groups in E1 explains why SE did not colonize any treatment group more readily than another.

Table 4.4 displays mean LG *Salmonella* counts observed during E2 between groups and between sampling days. No differences were observed between groups or sampling days due to > 75% of samples falling below the limit of detection of spot plating and many negative samples upon enrichment.

The prevalence of *Salmonella* during E2 was additionally analyzed upon enrichment of LG samples (Table 4.5). Differences were observed between SF with 5% positive samples versus 20% in PB (P=0.043) and 35% in SB (P=0.001). Differences were also observed between WC and SB with 15% positive in WC (P=0.046) and between SB and UC with 15% (P=0.039). The UC, SF, and WC groups were observed to be lower in *Salmonella* prevalence versus SB. Williams et al (2012) observed that the acidification of litter achieved by products like SB can lead to better survivability of *Salmonella,* which may explain the differences observed between SB and the SF and WC groups. It is unclear whether the differences observed between SF and other groups were due to the antimicrobial activity of SF or by some change in conditions of the litter that was not observed, which may have been a result of the application of SF. Further investigation is needed to confirm the antimicrobial abilities of SF, especially because SF was found to be similar in prevalence to the UC group. Additional measurements of litter conditions such as litter pH and ammonia emissions could help define the effect of SF on *Salmonella* prevalence.

Additionally, SE prevalence detected by BC sampling is summarized in Table 4.6. No differences were observed between groups or sampling days with UC pens at 47%, PB at 56%, SB at 69%, SF at 50%, and WC at 67% positive. BC have been observed to be the most sensitive method for *Salmonella* spp. detection in litter versus other methods (Caldwell et al., 1998; Skov et al., 1999; McCrea et al., 2005; Buhr et al., 2007; Talorico et al., 2021) so this method is more indicative of prevalence of SE within the pen on the surface of litter versus the LG method, which is more representative of the prevalence of SE on and under the surface of the areas sampled (under feeder, under waterlines, and in between). The lack of differences between groups in BC suggests that overall SE prevalence is not affected by any litter treatment.

The comparison of mean litter moisture content between treatment groups during E2 is displayed in Figure 4.3. No differences were observed between groups with litter moisture. This removes the possibility of litter moisture becoming a confounding variable for bacterial survivability in the litter.

The temperature and relative humidity of the WC pile at 1 m and .5 m in depth is shown in Figures 4.4 and 4.5, respectively. Figure 4.5 shows the temperature of the pile at 1m deep exceeding the target temperature of 55℃ established by Jones & Martin in 2003, but this temperature was only maintained for approximately 36 hours. Figure 4.6 shows that the temperature at .5 m did not reach the target temperature of 55℃. This data shows that the core temperature of this pile was adequate to kill *Salmonella* spp. based on thermal death point of 55℃ for 1 hour reported by Jones & Martin in 2003. However, at more shallow depths this temperature was not achieved which allows for the possible survival of SE. This data suggests that adequate temperature for pasteurization could be met in these conditions, but more time is necessary to maintain these temperatures. Additionally, turning is necessary to ensure all litter

reaches the target temperature to kill SE, which was reached in the core of the piles, but not closer to the surface of the pile.

Experiment 3

The prevalence of *Salmonella* detected by BC during E3 was very low (Table 4.7). All LG samples during E3 were negative upon enrichment (data not shown). There were no significant differences between groups or sampling days in BC. Prevalence was observed to be 4% in UC pens, 12% in PB, 14% in SB, 8% in SF, and 3% in WC.

Sampling ceca of birds raised during E3 resulted in SE recovery from only one sample in the PB group (data not shown). All samples that were collected to detect the nalidixic acid resistant SE strain were also cultured to detect Nalidixic Acid Susceptible *Salmonella* (NS) to observe if other serovars of *Salmonella* were present in the environment and possibly taking precedence over the marker strain. NS was found in the ceca of 17% in PB, 17% in SB, 22% in SF, and 22% in WC birds (Table 4.8). This raises the question if the flock came from the hatchery infected with NS or if it had been introduced to the environment at some point. Additionally, this raises the question if this could affect the prevalence of Nalidixic acid resistant *Salmonella* in the ceca or litter by competitive exclusion of one serovar by another. It has been reported by one group that colonization of a subsequently introduced serovar is reduced when challenging with one serovar at 1 day in age before challenging with a subsequent serovar at 2 days (Pineda et al, 2021). If the non-marker strain *Salmonella* was present in the ceca of chicks from the hatchery, the colonization of the chicks by SE from the environment could be expected to be less likely or significantly reduced.

Nalidixic acid susceptible *Salmonella* (NS) was additionally recovered in LG and in BC samples during E3. Table 4.9 summarizes LG detection of NS during E3, no differences were observed

between treatment groups. Some differences were observed within treatment groups between sampling days. The SB group decreased from 62% positive pens on D-3 to 12% on D0, D4, and D11 (P=0.02). The SF group decreased from 75% on D-3 to 25% on D0 and D4 and then further to 12% (P=0.045). Differences were observed in WC between D-3 (62%) and D4, D7, and D11 (0%) (P=0.025). This data suggests that NS was prevalent in the environment during the flock grow out but decreased over time as birds aged and remained constant during downtime.

Displayed in Table 4.10 is the prevalence of NS detected by BC during E3. No significant differences were observed between treatment groups. The similarity in NS prevalence between groups in LG and BC suggests that the infection of the flock with NS was uniform and not introduced to one pen, treatment group, or area of the house. This suggests the hatchery could be the source of this non-marker strain *Salmonella* because a full clean out was performed before starting this study. If chicks coming in were colonized or contaminated with *Salmonella* spp., this would most likely make it equally prevalent in the environment of all pens. However, there were differences in some groups between D-10 and other sampling days. The SB treated pens decreased from 62% on D-10 to 12% on D-3 (P=0.039) and then to 0% on D0, D4, and D7 (P=0.025). This data reinforces differences seen in LG over time (Table 4.9). NS prevalence did decrease as the flock increased in age and stayed constant during downtime which is consistent with earlier findings.

Due to the inability to obtain chicks at hatcheries because of COVID-19 restrictions, chicks were unobtainable for a 15-week downtime, so SE and NS was monitored weekly until placement of the next flock (D104). The prevalence of SE detected by BC during the 15-week downtime after the third flock grow-out is summarized in Figure 4.6. No differences were observed between groups with prevalence observed at 18% in UC, 13% in PB, 14% in SB, 11%

in SF, and 16% in WC. There was a notable spike in prevalence on D47 within all groups with all groups having ≥50% prevalence (data not shown). No differences in conditions were noted at this time so an explanation is unknown for this spike. SE was not recovered during the 15-week downtime in LG sampling due to the lack of sensitivity in this method (data not shown). This data demonstrates that SE is very low in prevalence in the environment after raising two flocks in the same environment as the seeder flock. However, SE did persist throughout the long downtime.

To summarize the detection of NS by BC and LG during the 15-week downtime is shown in Figures 4.7 and 4.8; no differences were observed between groups. There was a notable spike in BC NS prevalence on D47 within all groups with all groups having ≥50% prevalence which was similar to what was observed in SE prevalence, no changes in conditions were documented to explain this spike (data not shown). Data from Figure 4.7 and 4.8 further suggests that the infection of the flock with NS was uniform and not introduced to one pen, treatment group, or area of the house..

The means observed between groups in litter moisture during E3 and during the additional downtime are shown in Figure 4.9. Litter moisture percentage was 17.78% in UC, 18.69% in PB, 18.59% in SB, 19.03% in SF, and 17.87% in WC pens. Differences were observed between WC and PB, SB, and SF ($P \leq 0.01$), UC was similar to WC, PB, and SF. Although the SF group was observed to have higher litter moisture content than other groups, this difference did not affect the prevalence of SE or NS *Salmonella* in E3 (Figure 4.7 & 4.8). Litter moisture did decrease constantly over the extended downtime in all groups, but prevalence of SE and NS *Salmonella* was not affected.

The observed temperature and humidity measurements from 0.5 m deep and 1 m deep in the WC pile are shown in Figure 4.10 and Figure 4.11, respectively. The temperature reached the target temperature of 55°C for approximately 4 days at the depth of .5 m and for approximately 6 days at 1 m deep, verifying that the pile reached the target temperature of 55°C and met the thermal death point for *Salmonella* spp. described by Jones and Martin in 2003 at 1 m and 0.5 m in depth.

Experiment 4

Tables 4.11 and 4.12 summarize the detection of NS by LG and BC during E4, respectively. Pens with fresh bedding on the ends of each row were added with the objective of seeing if fresh bedding would become infected by NS *Salmonella* from incoming birds. Pens designated for FB were sampled with BC to ensure they were *Salmonella* spp. free before laying down fresh bedding, which was also confirmed to be *Salmonella* spp. free by performing LG and BC before the placement of chicks. Differences in LG were observed between the eight added FB and all other groups. The prevalence of NS detected in each treatment after the fourth flock grow-out was 5% in UC (P=0.023), 2% in PB (P=0.007), 5% in SB (P=0.023), 0% in SF (P=0.002), 3% in WC (P=0.014), and 22% in FB (Table 4.11). The FB group was observed to have higher NS prevalence than all other groups, which further confirms the findings that litter previously inhabited by birds inhibits the spread of *Salmonella* spp. versus the use of fresh bedding (Olesiuk et al., 1971), which was observed in this case regardless of treatment. No differences were observed between groups or between sampling days in BC (Table 4.12). Nalidixic acid resistant *Salmonella* (SE) was not detected in the environment during E4 and all ceca samples were negative for *Salmonella* spp. (data not shown).

The litter collected during E4 was enumerated for total aerobic bacteria to investigate the possibility of these treatments affecting the bacterial load of aerobic bacteria in the litter. Differences between sampling days were observed in total aerobic bacteria counts within groups (Table 4.13). Differences in counts between the UC pens were between D6 and D0, D3, D9, and D13 (P=0.01). In PB pens, D6 aerobic counts were greater than D0 and D9 (P=0.035). Aerobic counts in SB pens on D6 were greater than counts on D0, D3, D9, and D13 (P=0.043). Differences in counts within the SF group were between D6 and D0, D3, D9, and D13 (P=0.017). Aerobic counts on D6 in the WC group were greater than D3 and D9 (P=0.019). In the FB group, counts on D6 were greater than D0, D3, D9, and D13 (P<0.01). No changes in conditions were documented that could increase the total aerobic bacteria in the litter. It could be hypothesized that the removal of birds from the pens allowed for some growth of aerobic bacteria due to the lack of competitions with other microbes from the feces of broilers, although numbers decreased after D6 to levels similar to counts observed on D0 and D3. Numbers may have returned to normal after nutrients for survival depleted as litter remained undisturbed for more time.

Differences in the average total aerobic bacterial counts were observed between SF and SB, UC, PB, and FB (P=0.015) (Figure 4.12). The average total aerobic counts observed during E4 were 7.57log10(cfu/g) in UC, 7.61 in PB, 7.52 in SB, 7.01 in SF, 7.42 in WC, and 7.68 in FB. The differences observed in mean aerobic counts between SF and other groups demonstrates a small reduction of the bacterial load of reused litter with the application of SF. The use of SB did not affect total aerobic bacteria after four reuses of litter which differs from the observations of Pope & Cherry in 2000 who reported a decrease in aerobic bacteria after applying SB to litter that was used once.

The comparison of average *E. coli* counts during each sampling day within each treatment is displayed in Table 4.14. Differences were observed in each group with gradual reductions in counts over the downtime. Significant differences within the UC group were between D0 and D6, D9, and D13, and between D3 and D13 (P=0.022). Differences in the PB group were between D0 and D6, D0 and D9, D0 and D13 (P=0.01), D3 and D13 (P<0.001), and D6 and D13 (P=0.004). *E. coli* counts in the SB group also decreased over time with differences between D0 and D3, D6, D9, and D13 (P=0.01), and between D6 and D13 (P=0.026). Counts in SF treated pens were different between D0 and D6, D9, D13 (P<0.001), and between D3 and D6, D9, and D13 (P=0.048). Differences in the WC group were observed between D0 and D3, D6, D9, D13 (P=0.017), and between D3 and D9 (P=0.003), and D13 (P=0.001). Additional differences were observed in the FB group between D0 and D9 and D13 (P<0.01), and between D3 and D13, and D6 and D13 (P=0.01). It was observed that *E. coli* counts gradually decreased during downtime in each group, which could be due to the conditions of litter becoming suboptimal for the survival of coliforms or that birds are not present to excrete and spread more coliforms. Reductions of *E. coli* counts between 3 to 5 log10 have been reported when litter is left sitting in empty broiler houses between 2 to 16 weeks, so reduction in *E. coli* counts over this two-week downtime was expected (Kelley et al., 1994).

Figure 4.13 summarizes the average *E. coli* counts observed during E4. Differences between groups were between UC and FB (P=0.037), SF (P=0.049), and between SB and FB (P=0.002), between SF and FB and PB (P=0.001), and between WC and FB (P=0.004). The FB group had the highest average *E. coli* counts during E4 out of all groups, similar results were seen with NS prevalence in LG during E4. The SF and WC groups had the lowest average *E. coli* counts during E4. The use of SB did not appear to affect the *E. coli* counts in litter versus other

treatments, which does not agree with reports from Pope & Cherry in 2000, although counts were observed after multiple reuses of litter in this study versus one reuse in Pope & Cherry's study. Additionally, SF counts were also lower than UC counts, showing a difference between reused litter that was untreated and reused litter that had been treated with SF three times. These observations do correlate with previous observations of the SF and WC groups having lower SE prevalence in LG during E2 (Table 4.5) and the observation of SF having the lowest total Aerobic Counts in E4 (Figure 4.12). This data implicates the antimicrobial activity of SF and WC versus other treatments.

The average total coliform counts of LG samples were also analyzed in E4 (Figure 4.14). Statistical differences were observed between UC and SF (P=0.037), PB and SF (P=0.005), and between FB and SB, SF, and WC (P=0.01). The observations made in total coliform counts were similar to observations made in average *E. coli* and total aerobic counts during E4 (Figure 4.12 & 4.13). Differences between SF and other groups again provides some bases for the use of SF on poultry litter to reduce bacterial load, however additional investigation is needed to verify if SF has any antimicrobial effect against *Salmonella* spp.

The mean litter moisture percentage for each treatment during E4 can be referenced on Figure 4.15. Litter moisture percentage was observed to be 22.9% in UC, 23.8% in PB, 24.5% in SB, 26.2% in SF, 24.0% in WC, and 24.2% in FB. The mean litter moisture percentage in SF was observed to be significantly greater than in UC pens (P=0.023), no other differences were observed. Although SF was found to have the highest litter moisture percentage, SF still displayed small reductions of total aerobic bacteria, *E. coli*, and in total coliforms (Figures 4.12-14). Although higher litter moisture may contribute to better bacterial survivability (Carr et al., 1995; Payne et al., 2007; Turnbull & Snoeyenbos, 1973), this was not observed in this study with

the use of the liquid SF treatment and differences in litter moisture between SF and other groups was minimal. This data suggests that liquid antimicrobials could be utilized to reduce the microbial load and *Salmonella* spp. prevalence in reused poultry litter, regardless of the increase of litter moisture. The use of metam-sodium, a liquid soil fumigant, was reported to reduce aerobic bacterial counts in poultry litter by its production of by-products (Macklin & Krehling, 2010). Macklin & Krehling (2010) saw higher reduction in aerobic bacteria with higher application rates of metam-sodium and controlled their study for litter moisture, this suggests further investigation of the use of SF could possibly reveal higher reduction of the bacterial load of litter with some refinement to methods.

The marker strain did persist in the environment for over 230 days in all treatment groups. This agrees with the findings of two groups that SE can persist in reused litter for months to years, although SE prevalence does decrease with each reuse of litter (Davies & Wray, 1996; Roll et al., 2014). No treatment was consistently effective in reducing SE prevalence although some differences were observed in SF and WC groups in E2 LG, but these treatments were not different from the UC group (Table 4.5). Due to these observations the use of any of these treatments for *Salmonella* spp. control is inconclusive, although some decreases in antimicrobial load of the litter were seen in E4 with the use of SF and WC which warrants more investigation (Figures 4.12, 4.13, and 4.14). Additionally, the turning of the WC piles would certainly aid in decreasing microbial load of reused litter by exposing a larger proportion of the litter to target temperatures that were reached in the core of the piles. It was also observed that the third flock (E3) was colonized with a considerable proportion of NS at market age and NS was moderately prevalent in the environment, which could have affected the prevalence of SE by NS excluding SE in the environment or GI tract of birds. This occurrence did allow for the observation of each

treatment when the environment was naturally infected with non-marker strain *Salmonella* spp. The FB pens were more easily infected with NS versus others, although no differences were seen in other treatments. The prevalence of NS in the FB pens that were found to be *Salmonella* spp. free suggests that wild-type *Salmonella* spp. may have been coming in with chicks from the hatchery.

CONCLUSIONS AND APPLICATIONS

- Treatments did not have any significant effect on the transmission of SE from one flock to the next when comparing SE recovery from the ceca of market age birds.
- No treatment was consistently effective in controlling *Salmonella* spp. prevalence in reused litter, although some differences were observed at certain times
- The antimicrobial capabilities of Windrow Composting were further confirmed in LG SE prevalence observed in E2 and in *E. coli* counts observed in E4, although complete removal of *Salmonella* was not achieved and no differences were observed in BC sampling.
- The use of SB was observed to increase SE prevalence in LG versus the UC group in E2, although no other differences were observed in *Salmonella* spp. prevalence or on the bacterial load of litter during E4
- Observed SE prevalence in LG during E2, Aerobic counts during E4, total coliform counts, and *E. coli* counts during E4 lead to the potential us of SF to reduce the bacterial load of litter. Although more research is needed due to no differences observed between treatment in *Salmonella* prevalence detected by BC and due to no difference between SF and UC in E2 LG.
- Liquid antimicrobials may be a viable option to reduce the bacterial load and prevalence of *Salmonella* spp. in reused poultry litter, but further investigation is needed.

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DATA

Table 4.1: E1 Ceca *Salmonella* Enteritidis Recovery

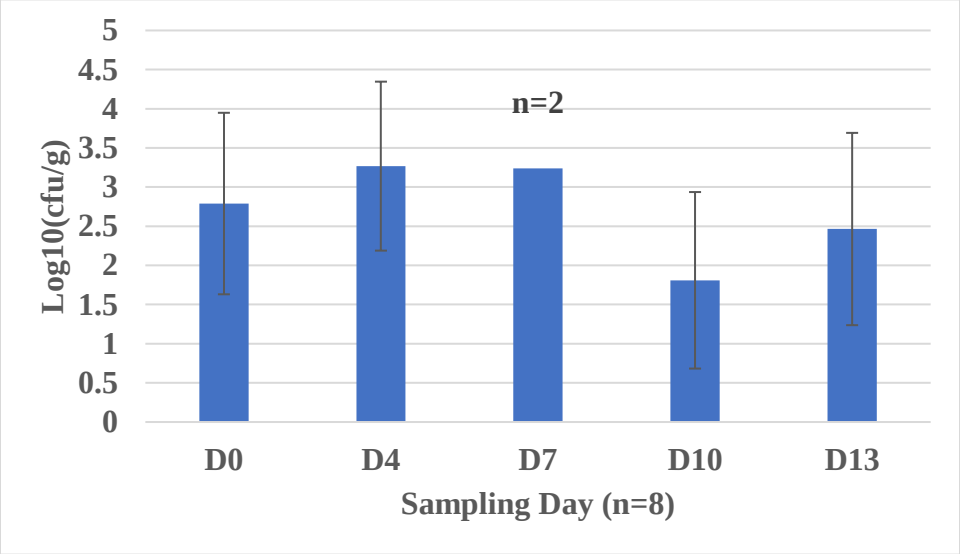
	UC	PB	SB	SF	WC
+	11/40	13/40	12/40	18/40	21/40
%	27[b]	32[ab]	30[b]	45[ab]	52[a]

[a-b] Values within a row with differing letters are significantly different ($P \leq 0.05$).

+ denotes the proportion of samples positive for *Salmonella*

% denotes the percentage of samples positive for *Salmonella*

Figure 4.1: E1 Windrow Compost Litter Grab *Salmonella* Enteritidis Counts

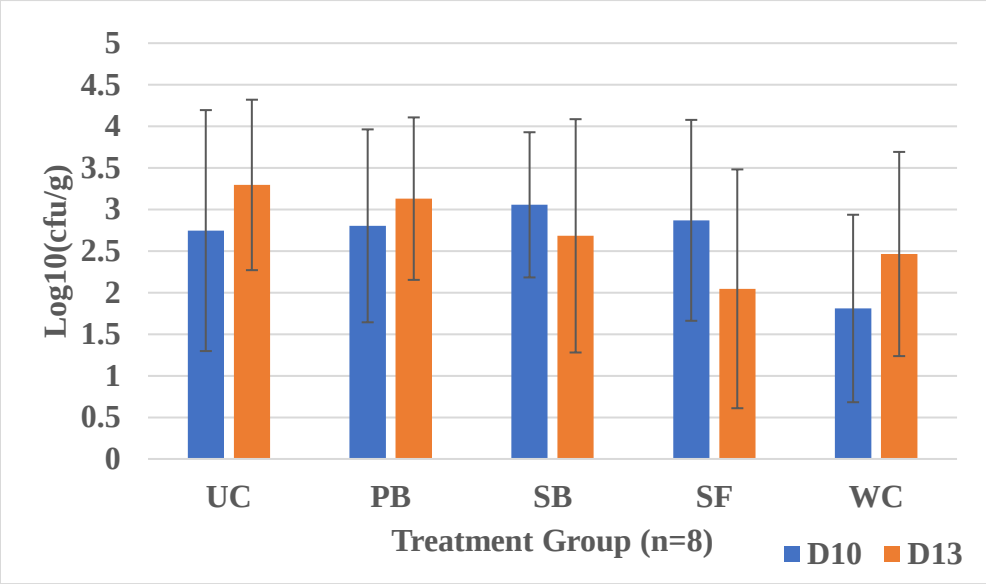


Description: This figure depicts Litter Grab *Salmonella* counts from D0 to D13 with the Windrow Compost pile formed after sampling on D4 and litter placed back into pens before sampling on D10.

Table 4.2: E1 Boot Cover *Salmonella* Enteritidis Prevalence

	UC	PB	SB	SF	WC
D0	8/8	8/8	8/8	7/8	8/8
D4	8/8	8/8	8/8	8/8	8/8
D7	8/8	7/8	8/8	7/8	N/A
D11	8/8	8/8	8/8	8/8	8/8
D13	8/8	8/8	8/8	8/8	8/8
Total	40/40	39/40	40/40	38/40	32/32
%	100	97	100	95	100

Figure 4.2: E1 D10 and D13 Litter Grab *Salmonella* Enteritidis Counts



Description: This figure depicts *Salmonella* Enteritidis counts by Litter Grab on D10 and D13. The sampling on D10 was before the application of Probiotic Blend, Sodium Bisulfate, and Sodium Formate Salts. Sampling on D13 was 3 hours after the application of the previously mentioned treatments.

Table 4.3: E2 Ceca *Salmonella* Enteritidis Recovery

	UC	PB	SB	SF	WC
+/-	0/40	3/40	1/40	0/40	1/40
%	0	7	2	0	2

+ denotes the proportion of samples positive for *Salmonella*

% denotes the percentage of samples positive for *Salmonella*

Table 4.4: E2 Litter Grab *Salmonella* Enteritidis Counts

	UC	PB	SB	SF	WC
D-10	0.00	0.44	0.43	0.00	0.30
D-3	0.00	0.25	0.90	0.13	0.25
D0	0.50	0.50	0.50	0.00	0.13
D4	0.13	0.38	0.25	0.00	0.00
D7	0.38	0.00	0.50	0.13	0.13
Avg. (n=40)	0.20	0.31	0.52	0.05	0.16

Table 4.5: E2 Litter Grab *Salmonella* Enteritidis Prevalence

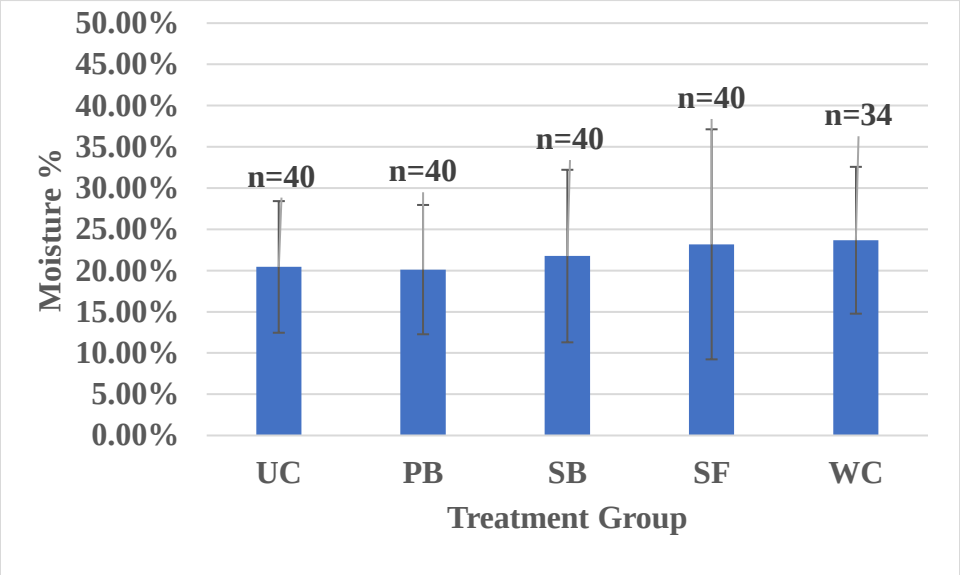
	UC	PB	SB	SF	WC
D-10	0/8	1/8	1/8	0/8	1/8
D-3	0/8	2/8	3/8	1/8	2/8
D0	2/8	2/8	4/8	0/8	1/8
D4	1/8	3/8	2/8	0/8	0/2
D7	3/8	0/8	4/8	1/8	1/8
Total	6/40	8/40	14/40	2/40	5/34
%	15^{bc}	20^{ab}	35^{a}	5^{c}	15^{bc}

$^{a-c}$ Values within a row with differing letters are significantly different ($P \leq 0.05$).

Table 4.6: E2 Boot Cover *Salmonella* Enteritidis Prevalence

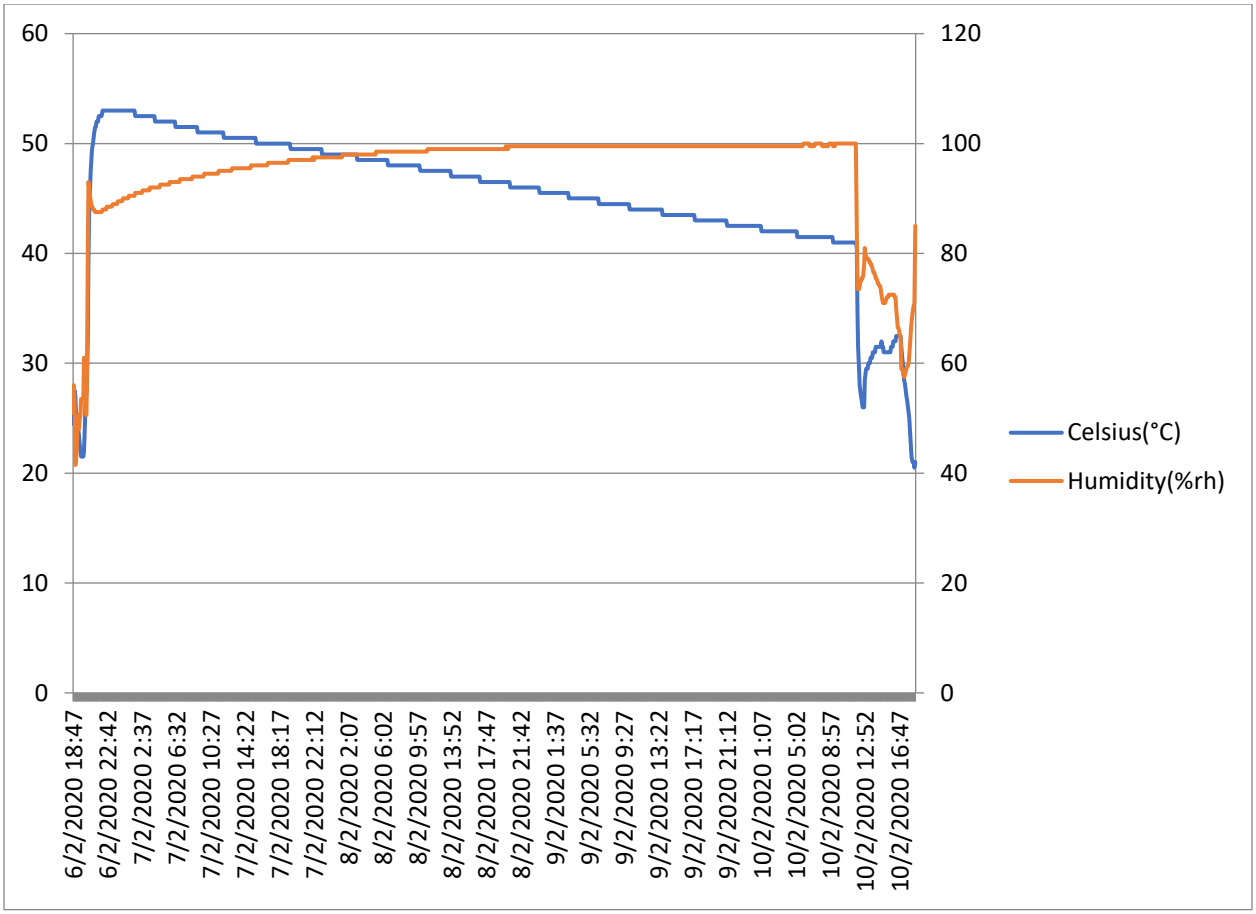
	UC	PB	SB	SF	WC
D-10	4/8	5/8	6/8	3/8	6/8
D-3	3/8	6/8	4/8	5/8	5/8
D4	5/8	5/8	5/8	2/8	N/A
D7	3/8	2/8	7/8	6/8	5/8
Total	15/32	18/32	22/32	16/32	14/24
%	47	56	69	50	58

Figure 4.3: E2 Average Litter Moisture Content



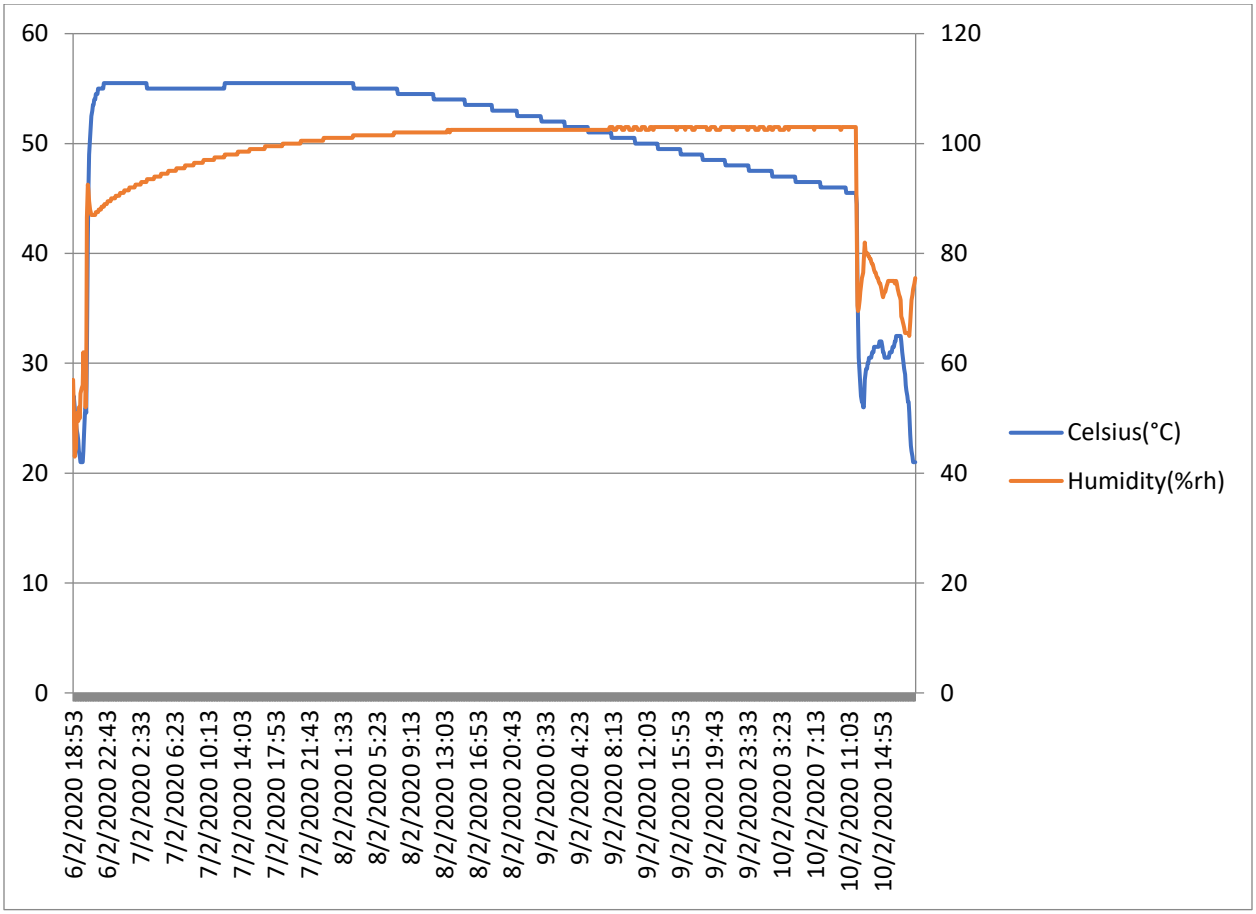
Description: This figure depicts the average litter moisture content of each treatment group during sampling for Experiment 2 (D-10-D7).

Figure 4.4: E2 Windrow Compost Pile .5 m Depth



Description: This figure summarizes Windrow Compost pile % relative humidity and temperature data collected from the data logger placed 0.5 m in depth during Experiment 2.

Figure 4.5: E2 Window Compost Pile 1 m Depth



Description: This figure summarizes Windrow Compost pile % relative humidity and temperature data collected from the data logger placed 1 m in depth during Experiment 2.

Table 4.7: E3 Boot Cover *Salmonella* Enteritidis Prevalence

	UC	PB	SB	SF	WC
D-10	0/8	0/8	1/8	0/8	1/8
D-3	0/8	1/8	2/8	2/8	0/8
D0	0/8	0/8	0/8	0/8	0/8
D4	1/8	3/8	2/8	1/8	N/A
D7	1/8	1/8	2/8	0/8	N/A
D11	0/8	1/8	0/8	1/8	0/8
Total	2/48	6/48	7/48	4/48	1/32
%	4	12	14	8	3

Table 4.8: E3 Ceca Nalidixic Acid Susceptible *Salmonella* Recovery

	UC	PB	SB	SF	WC
+	3/40	7/40	7/40	9/40	9/40
%	7	17	17	22	22

+ denotes the proportion of samples positive for *Salmonella*

% denotes the percentage of samples positive for *Salmonella*

Table 4.9: E3 Litter Grab Nalidixic Acid Susceptible *Salmonella* Prevalence

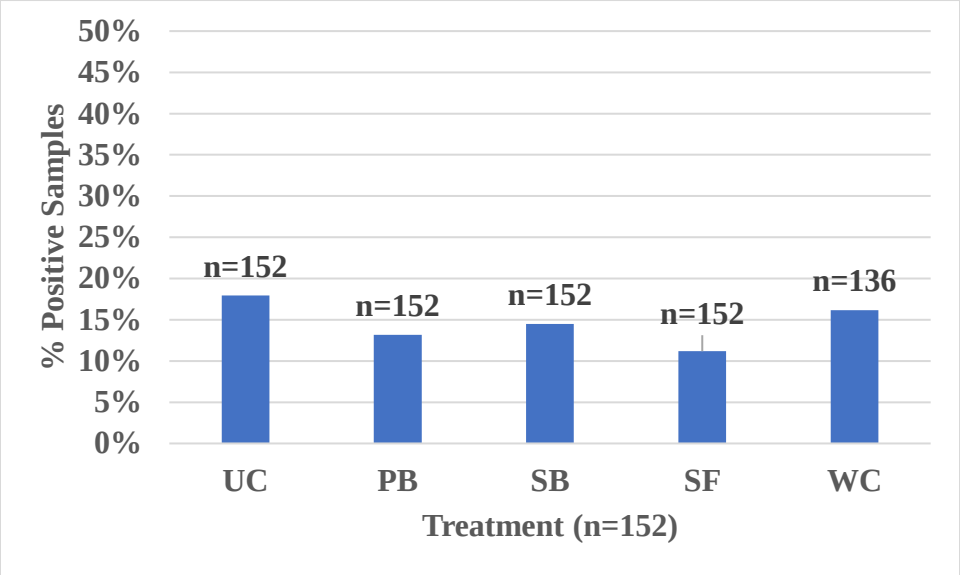
	UC	PB	SB	SF	WC
D-10	3/8	1/8	$3/8^{ab}$	$3/8^{ab}$	$4/8^{ab}$
D-3	4/8	3/8	$5/8^{a}$	$6/8^{a}$	$5/8^{a}$
D0	3/8	1/8	$1/8^{b}$	$2/8^{b}$	$3/8^{ab}$
D4	1/8	3/8	$1/8^{b}$	$2/8^{b}$	$0/2^{b}$
D7	3/8	0/8	$3/8^{ab}$	$3/8^{ab}$	$0/2^{b}$
D11	2/8	2/8	$1/8^{b}$	$1/8^{b}$	$0/8^{b}$
Total	16/48	10/48	14/48	17/48	12/34
%	33	21	29	35	35

Differences in SE prevalence between sampling days within each group are displayed by $^{a\text{-}b}$ values within columns, with differing letters showing statistical differences ($P \leq 0.05$).

Table 4.10: E3 Boot Cover Nalidixic Acid Susceptible *Salmonella* Prevalence

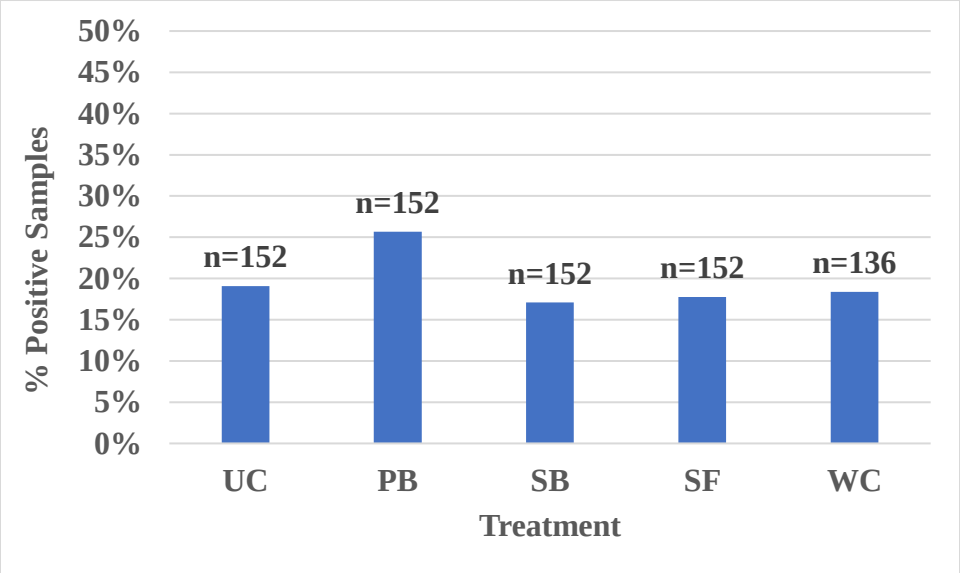
	UC	PB	SB	SF	WC
D-10	3/8	4/8	5/8	4/8	3/8
D-3	2/8	3/8	1/8	2/8	1/8
D0	0/8	0/8	0/8	0/8	0/8
D4	3/8	2/8	2/8	2/8	N/A
D7	1/8	0/8	0/8	0/8	N/A
D11	0/8	0/8	0/8	0/8	1/8
Total	9/48	9/48	8/48	8/48	5/32
%	19	19	17	17	16

Figure 4.6: E3 Downtime Boot Cover *Salmonella* Enteritidis Prevalence



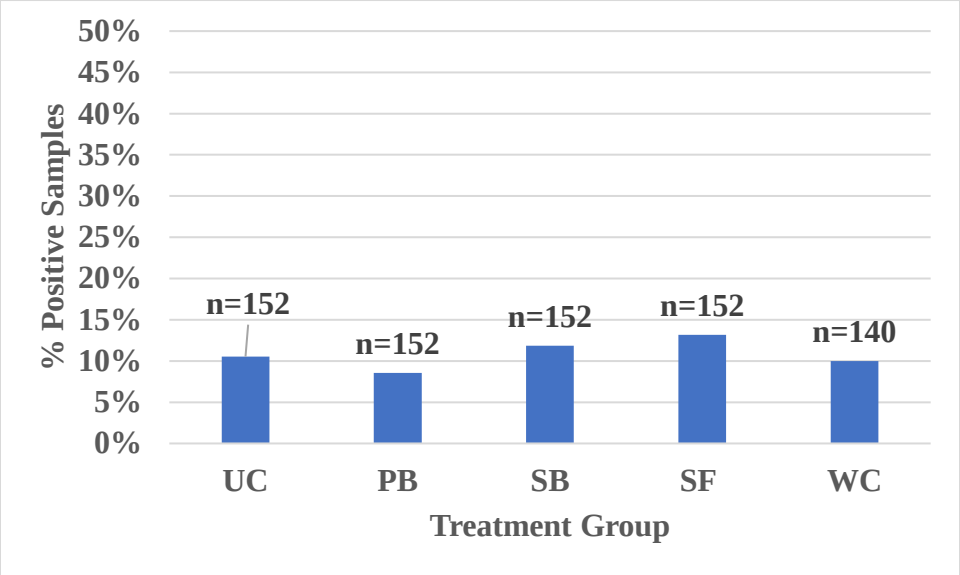
Description: This figure represents *Salmonella* Enteritidis prevalence detected by Boot Covers during the extended downtime that occurred after rearing of the flock for Experiment 3.

Figure 4.7: E3 Downtime Boot Cover Nalidixic Acid Susceptible *Salmonella* Prevalence



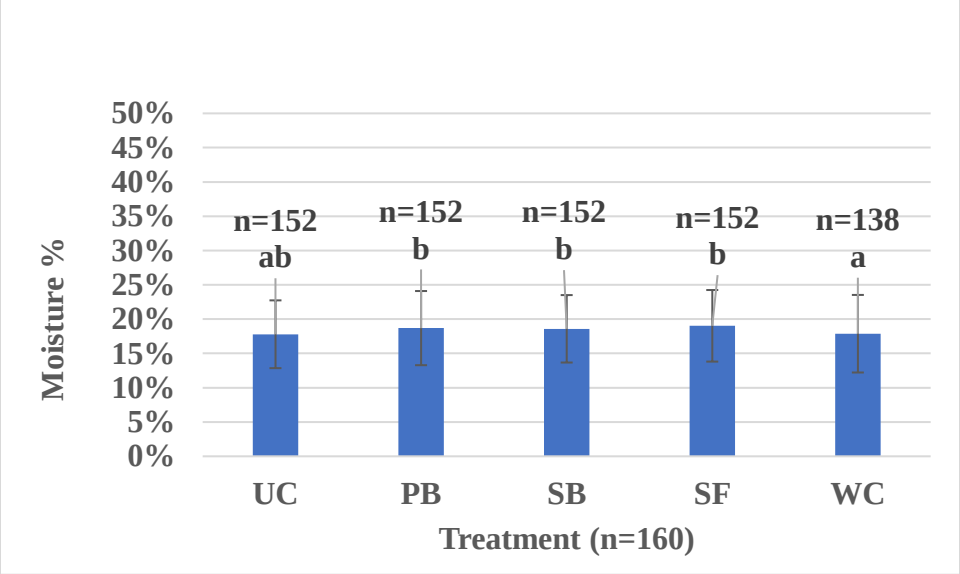
Description: This figure represents nalidixic acid susceptible *Salmonella* prevalence detected by Boot Covers during the extended downtime that occurred after rearing of the flock for Experiment 3.

Figure 4.8: E3 Downtime Litter Grab Nalidixic Acid Susceptible *Salmonella* Prevalence



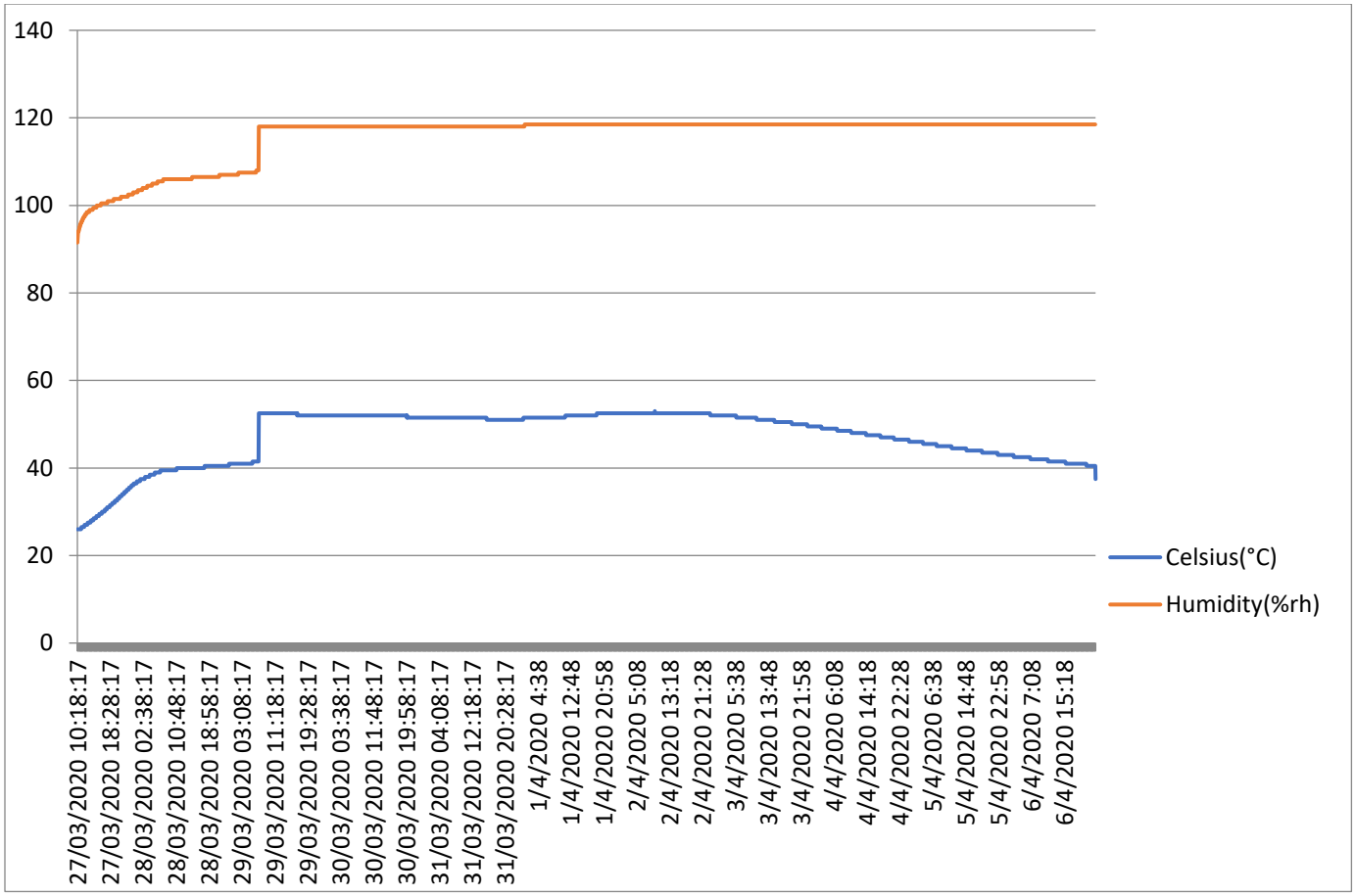
Description: This figure represents nalidixic acid susceptible *Salmonella* prevalence detected by Litter Grab during the extended downtime that occurred after rearing of the flock for Experiment 3.

Figure 4.9: E3 Average Litter Moisture



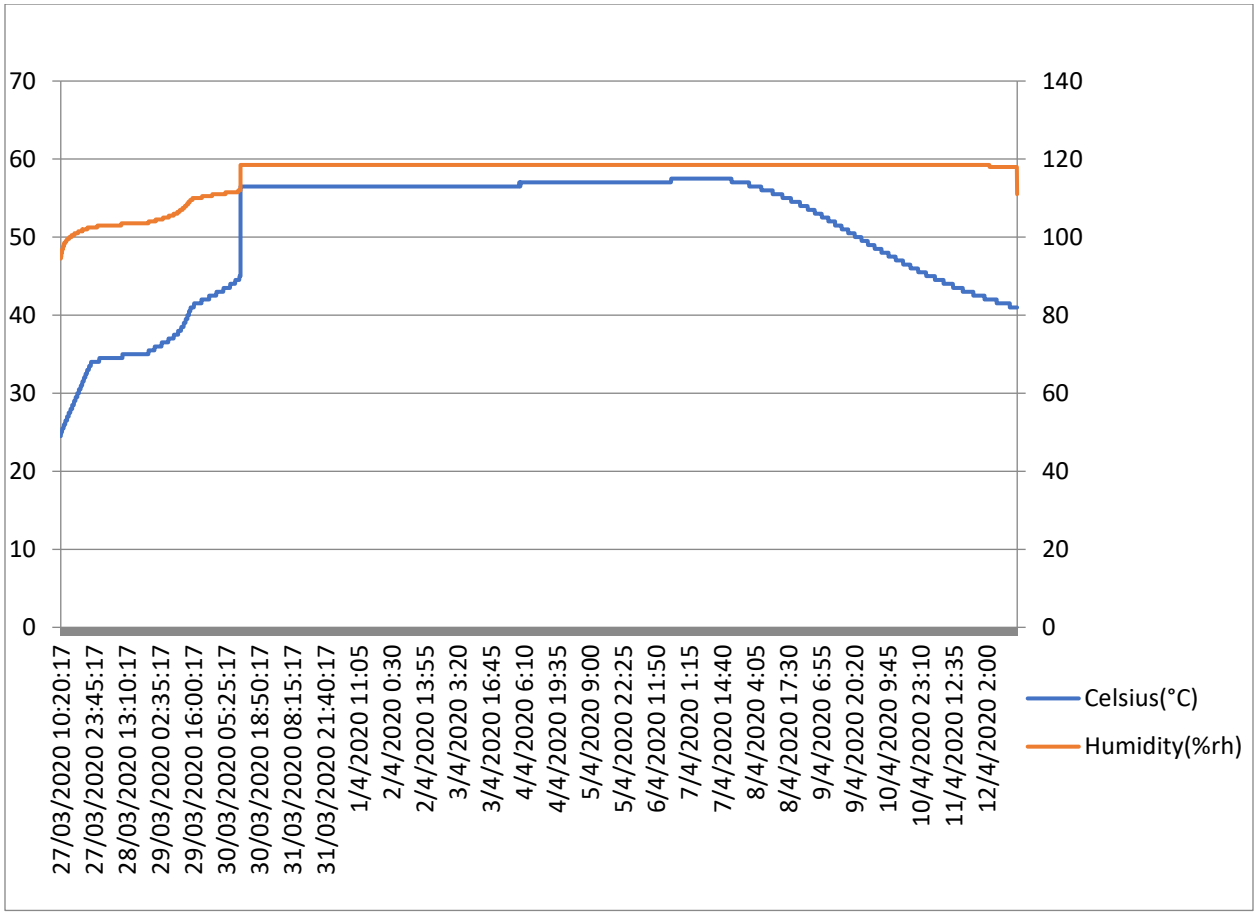
Description: This figure represents average litter moisture percentage during the extended downtime that occurred after rearing of the flock for Experiment 3. $^{a\text{-}b}$ Treatment groups with differing letters are significantly different ($P \leq 0.05$).

Figure 4.10: E3 Windrow Compost Pile .5 m Depth



Description: This figure summarizes Windrow Compost pile % relative humidity and temperature data collected from the data logger placed 0.5 m in depth during Experiment 3.

Figure 4.11: E3 Windrow Compost Pile 1 m Depth



Description: This figure summarizes Windrow Compost pile % relative humidity and temperature data collected from the data logger placed 1 m in depth during Experiment 2.

Table 4.11: E4 LG Nalidixic Acid Susceptible *Salmonella* Detection

	UC	PB	SB	SF	WC	FL
D0	0/8	0/8	1/8	0/8	1/8	0/8
D3	0/8	0/8	0/8	0/8	0/8	2/8
D6	0/8	1/8	0/8	0/8	0/2	3/8
D9	1/8	0/8	1/8	0/8	0/8	2/8
D13	1/8	0/8	0/8	0/8	0/8	2/8
Total	2/40	1/40	2/40	0/40	1/34	9/40
%	5^{b}	2^{b}	5^{b}	0^{b}	3^{b}	22^{a}

$^{a-b}$ Values in rows with differing letters are significantly different ($P \leq 0.05$).

Table 4.12: E4 BC Nalidixic Acid Susceptible *Salmonella* Detection

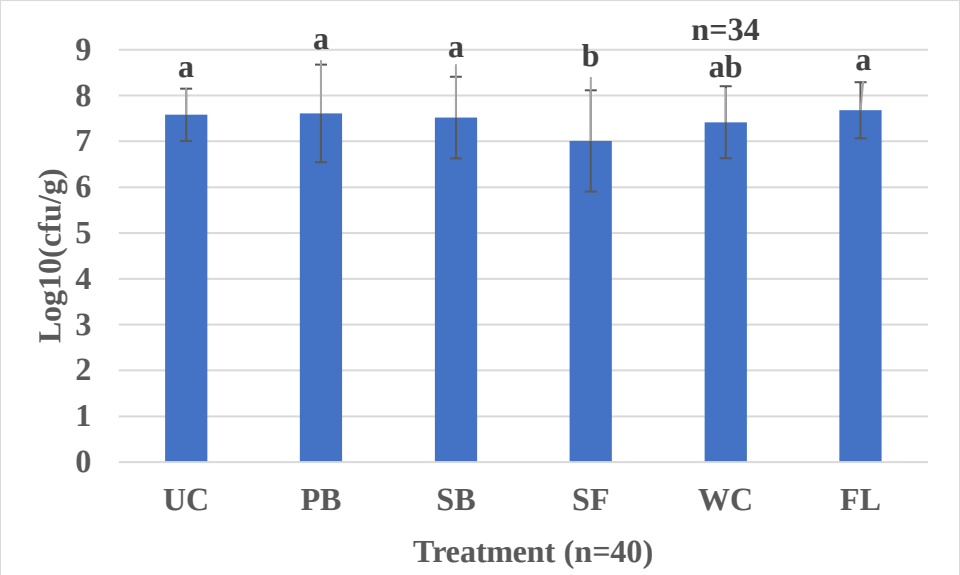
	UC	PB	SB	SF	WC	FL
D0	1/8	3/8	2/8	0/8	3/8	1/8
D3	0/8	2/8	0/8	0/8	0/8	2/8
D6	3/8	4/8	2/8	4/8	N/A	4/8
D9	2/8	1/8	3/8	3/8	1/8	2/8
D13	2/8	2/8	0/8	1/8	4/8	4/8
Total	8/40	12/40	7/40	8/40	8/32	13/40
%	20	30	17	20	25	32

Table 4.13: E4 LG Total Aerobic Bacteria Counts

	UC	PB	SB	SF	WC	FL
D0	7.51^{b}	7.21^{b}	7.51^{b}	6.73^{b}	7.61^{ab}	7.68^{b}
D3	7.54^{b}	7.53^{ab}	7.29^{b}	7.17^{b}	7.33^{b}	7.44^{b}
D6	8.29^{a}	8.63^{a}	8.55^{a}	8.47^{a}	8.39^{a}	8.52^{a}
D9	7.50^{b}	6.90^{b}	7.48^{b}	6.61^{b}	6.81^{b}	7.51^{b}
D13	7.09^{b}	7.74^{ab}	6.76^{b}	6.09^{b}	7.67^{ab}	7.22^{b}

$^{a-b}$ Values within columns with differing letters are significantly different ($P \leq 0.05$).

Figure 4.12: E4 Litter Grab Average Total Aerobic Bacteria Counts



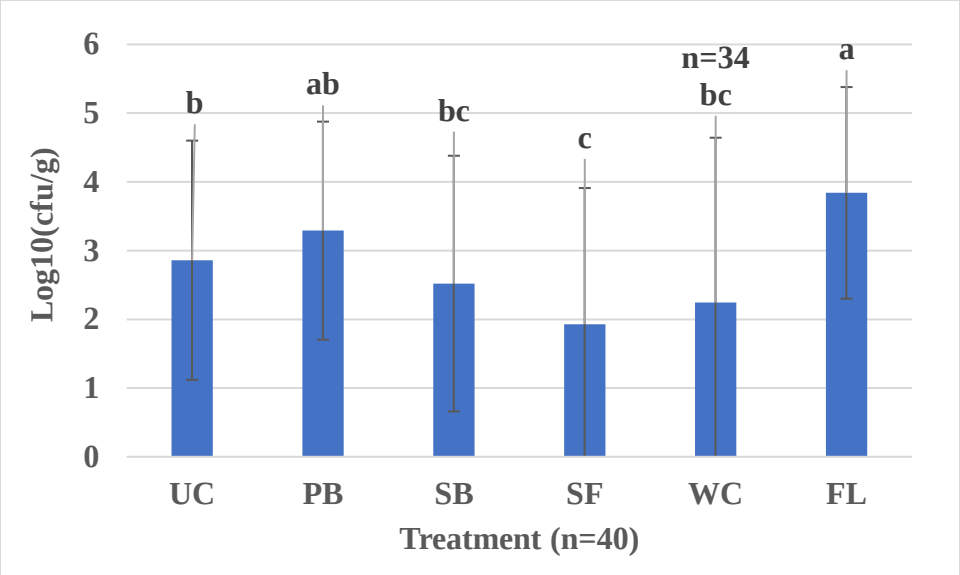
Description: This Figure summarizes average litter grab total aerobic bacterial counts collected during Experiment 4 (D0-D13). $^{a-b}$ Treatment groups with differing letters are significantly different ($P \leq 0.05$).

Table 4.14: E4 Litter Grab *E. coli* Counts

	UC	PB	SB	SF	WC	FL
D0	4.75^{a}	5.11^{a}	5.05^{a}	4.45^{a}	5.38^{a}	5.45^{a}
D3	3.75^{ab}	3.98^{ab}	1.88^{bc}	3.10^{a}	3.02^{b}	4.24^{ab}
D6	2.35^{bc}	3.36^{b}	2.86^{b}	1.43^{b}	2.06^{bc}	4.12^{ab}
D9	1.91^{bc}	2.55^{bc}	1.94^{bc}	0.50^{b}	0.50^{c}	3.24^{bc}
D13	1.60^{c}	1.42^{c}	0.89^{c}	0.12^{b}	0.12^{c}	2.15^{c}

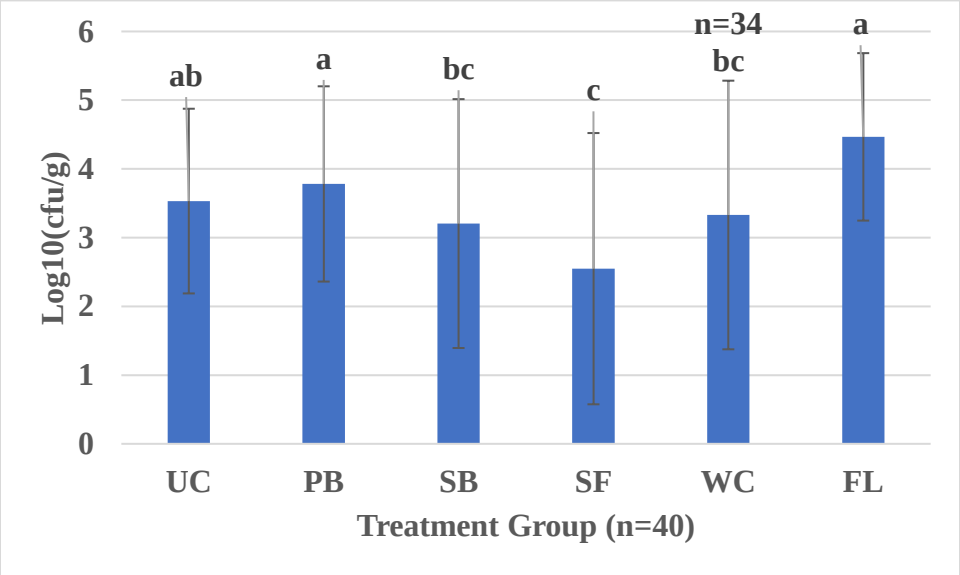
$^{a-c}$ Values within columns with differing letters are significantly different ($P \leq 0.05$).

Figure 4.13: E4 Litter Grab Average *E. coli* LG Counts



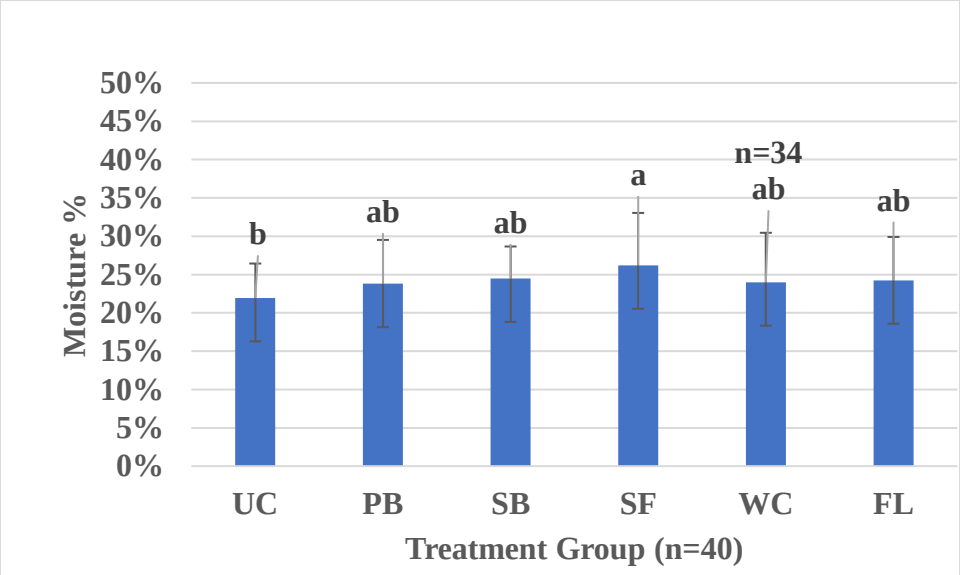
Description: This Figure depicts the average litter grab *Escherichia coli* counts collected during Experiment 4 (D0-D13). $^{a\text{-}b}$ Treatment groups with differing letters are significantly different ($P \leq 0.05$).

Figure 4.14: E4 Litter Grab Average Total Coliform Counts



Description: This Figure depicts the average litter grab total coliform counts collected during Experiment 4 (D0-D13). $^{a\text{-}b}$ Treatment groups with differing letters are significantly different ($P \leq 0.05$).

Figure 4.15: E4 Average Litter Moisture



Description: This Figure depicts the average litter moisture percentage during Experiment 4 (D0-D13). $^{a\text{-}b}$ Treatment groups with differing letters are significantly different ($P \leq 0.05$).

Chapter 5:

The Effect of Co-challenging with *Salmonella* Enteritidis and *Salmonella* Kentucky on the Colonization of the Broiler GI Tract

By A.A. Talorico

SUMMARY

The prevalence of *Salmonella* Enteritidis (SE) in all steps of poultry production remains a challenge in the industry. The prevalence of *Salmonella* Kentucky (SK) is also well known in poultry production; however, SK has not been known to be as pathogenic to humans as SE. This study aims to examine if one serovar of *Salmonella* spp. can exclude another in the gastrointestinal tracts of broilers. Chicks were challenged with either 10^7cfu of nalidixic acid resistant SE on the day after flock placement (D1) (SE), 10^7 CFU of SK at 5 days of age (SK), 10^7cfu of SE (D1 followed by 10^7cfu of SK on D7 (SE/SK), and 10^7cfu SK at D5 followed by 10^7cfu of SE at D7 (SK/SE). Five birds per pen were randomly selected for necropsy weekly which will be referred to as Experiment 1 (E1). Cecal content was collected to enumerate for nalidixic acid resistant *Salmonella* (NR), which represents SE and nalidixic acid sensitive *Salmonella* (NS) to represent SK. Experiment 2 involved the same sampling schedule as E1 with unchallenged birds raised in the same environment. In E1 differences in NS counts were observed, with SK having higher counts than SE/SK ($P \leq 0.026$). In E1, SE NR counts were greater than SK/SE group ($P \leq 0.04$). Additional differences were seen in prevalence with SK having higher NS prevalence versus the SE/SK group, and greater than SK/SE ($P \leq 0.015$). Differences in NR were also present with SE having higher prevalence than SK/SE ($P < 0.03$). In E2 only NS was found in the ceca of birds and no notable differences were observed. The reduction in colonization by one serovar by introducing another serovar firstly suggests that one serovar of *Salmonella* spp. can partially exclude the colonization of another subsequently introduced serovar. This finding leads to the possibility of developing a *Salmonella* surrogate to exclude *Salmonella* spp. from the gastrointestinal tract of broilers.

DESCRIPTION OF PROBLEM

Nontyphoidal *Salmonella* is a significant public health concern in the United States with Salmonellosis responsible for a considerable proportion of foodborne illness cases. In 2016, 53 state and regional public health laboratories reported 46,623 cases of culture-confirmed *Salmonella* infections to the Laboratory-Based Enteric Disease Surveillance System with *Salmonella* Enteritidis (SE) being the most frequently reported serotype (CDC, 2016). A study in 2012 by Batz, Hoffman, and Morris compiled data implicating *S. enterica* and poultry products as the fourth highest pathogen/food product combination in disease burden (Batz et al., 2012). *Salmonella* spp. colonizes broiler chickens by oral or intracloacal uptake of contaminated litter or by aerosolized *Salmonella* spp. in dust. This model of colonization was established by challenging birds by orally, intratracheally, or by aerosolizing contaminated poultry dust and then recovering *Salmonella* from the ceca of birds (Barrow 1991; Nakamura et al., 1995 ; Gast, Mitchell, and Holt, 1998; Bailey et al., 2006; Chadwick et al., 2020) The ceca is recognized as the most readily colonized organ in broilers and is commonly cultured to determine the colonization of broilers with *Salmonella* spp. (Fanelli et al., 1971; Snoeyenbos et al., 1982; Chadwick et al., 2020).

Methods for the exclusion of *Salmonella* from the GI tract of broilers have been investigated by many groups. An early method investigated by Nurmi et al. in 1973 involved introducing adult chicken gut contents for competitive exclusion (CE) to the crops of chicks to exclude the colonization of *Salmonella infantis* after challenge (Nurmi and Rantala, 1973. This method was successful in reducing *Salmonella infantis* colonization of the crop, small intestine, and ceca (Nurmi and Rantala 1973). Other groups have expanded on this finding to introduce more microbiologically defined CE cultures with varying species and have seen similar results in

excluding *Salmonella* Enteritidis (SE) (La Ragione and Woodward 2003; Nakamura et al., 2002; Nisbet 2002; Zhang and Doyle 2007; Vilà et al., 2009).

It has been observed in an epidemiological study in Wales, UK, and in the US that SE has taken place of the ecological niche of *Salmonella* gallinarum since the early 20th century (Rabsch et al., 2000). This observation leads to the question if one serovar of *Salmonella* could exclude another in the GI tract of animals or in the environment. *Salmonella* Kentucky (SK) is one of the most frequently isolated serovars in poultry products that also causes a very low incidence of clinical disease in humans (CDC, 2016). With SK in high prevalence in poultry and low pathogenicity in humans, it would be beneficial to see if it could exclude more pathogenic serovars, such as SE which was confirmed in 16.8% of cases of Salmonellosis in 2016 (CDC, 2016). Pineda et al examined co-challenging chicks at 1 day of age with SE, SK, and/or *Salmonella* Typhimurium in 2021 and observed that whichever serovar was administered first excluded the subsequent serovar. The objective of this study was to co-challenge chicks with SE and SK to observe if one serovar excludes another in the ceca of broilers and in the litter between broiler flocks. Additionally, a second flock was raised in the same environment to observe the transmission of both serovars from one flock to the next.

MATERIALS AND METHODS

Experiment 1 Design

In Experiment 1 (E1), 1000 commercially obtained straight run broiler chicks were spread over 20 1.52 m^2 pens (50 birds/pen) onto fresh pine shavings. Pens were arranged into two rows of ten pens with rows on each side of an aisle. The birds were fed a standard starter, grower, and finisher diet that met or exceeded the NRC suggested nutritional requirements of broilers (NRC, 1994). Four treatments were assigned to five pens each. Treatments consisted of

all birds in corresponding pens challenged with either 10^7cfu of nalidixic acid resistant *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) on the day after flock placement (D1) (SE), 10^7 CFU of *Salmonella enterica* subspecies *enterica* serovar Kentucky (SK) at 5 days of age (SK), 10^7cfu of SE (D1 followed by 10^7cfu of SK on D7 (SE/SK), and 10^7cfu SK at D5 followed by 10^7cfu of SE at D7 (SK/SE) (Table 5.1). Birds were challenged with SK on D5 versus challenging on D1 due to an error of preparation of the inoculum that would have been used on D1. Five birds per pen were randomly selected for necropsy on Day 14 (D14), Day 21 (D21), Day 28 (D28), Day 35 (D35), Day 42 (D42), and Day 49 (D49) with the remaining birds terminated at D49. Cecal content was collected to enumerate for nalidixic acid resistant *Salmonella* (NR) and nalidixic acid sensitive *Salmonella* (NS). Additionally, litter was collected from each pen by litter grab method (LG) on D49 and after 14 days of downtime (D63) to enumerate for NR and NS and to enrich for the presence of NR or NS. NR was quantified theoretically by taking the difference between counts observed on XLT4 (Criterion Dehydrated Culture Media, Hardy Diagnostics, Santa Maria, CA) and counts observed on XLT4+ plates containing 100 µg/ml of nalidixic acid (Alfa Aesar, Haverhill, MA) and 15 µg/ml of novobiocin (Alfa Aesar) (XLT4+) within the same sample. In the incidence of greater counts observed on XLT4 versus XLT4+, the difference observed was reported as NS counts.

Experiment 2 Design

For Experiment 2 (E2) chicks were placed on the same litter that was used for E1 at the same stocking density (50 birds/pen) after 14 days of downtime. Birds were necropsied on the same days as E1 with identical methods. LG was also performed on Day 49 and Day 63 utilizing the methods described below.

Challenge Methods

Chicks were challenged with a 0.25 ml suspension of SK or SE containing approximately 4 x 10^7 cfu/ml utilizing a 1cc tuberculin syringes by oral gavage. The isolates were prepared from a culture stored at -80°C and then transferred onto tryptic soy agar with 5% sheep's blood (VWR Scientific, Radnor, PA) and incubated at 37°C for 18-24 hours. The isolates were confirmed as *Salmonella* using Difco Salmonella O Antiserum Poly A- I & Vi (Becton, Dickinson, and Company, Sparks, MD). One colony was then streaked onto Xylose Lysine Tergitol 4 (XLT4) (Criterion Dehydrated Culture Media, Hardy Diagnostics, Santa Maria, CA) for the SK isolate and the SE isolate was streaked onto XLT4+ plates containing 100 µg/ml of nalidixic acid (Alfa Aesar, Haverhill, MA) and 15 µg/ml of novobiocin (Alfa Aesar) (XLT4+) and both plate types were incubated for 18±1 hours at 37°C. SE and SK inoculum were prepared via overnight culture by inoculating 50ml of Brain Heart Infusion Broth (BHIB, Hardy Diagnostics) with one colony of SE isolated from XLT4+ or one colony of SK isolated on XLT4 and the inoculated flasks were then placed in a New Brunswick Innova 4300 Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for 18-24 h with 200 revolutions per minute (RPM) at 37°C. BHIB overnight culture was then diluted 100x with 1x Phosphate Buffered Saline (PBS) (VWR Scientific) to prepare the proper challenge dose of 1x10^7 cfu/ml. Inoculum were confirmed by making dilutions to spread plate onto XLT4+ and XLT4. The SE inoculum on D1 and D7 were observed to contain 2.4 x 10^7cfu/ml and 1.25 x 10^7cfu/ml, respectively. While the SK inoculum on D5 and D7 were observed to contain 1.2 x 10^7cfu/ml and 1.5 x 10^7cfu/ml, respectively.

Sampling Methods

Five birds per pen were randomly selected for ceca collection. The selected birds were euthanized and ceca aseptically collected, sliced at both ends, and content emptied into 50ml

conical vials (VWR Scientific). These were then set on ice prior to transport to the lab. Cecal content was then weighed and then serially diluted ten-fold by adding PBS to the vials, vortexing to homogenize the mixture, and then plated onto the media listed below. Cecal content samples were enumerated on XLT4+ and XLT4 by spot plating 10 µl onto grid plates (VWR Scientific) in triplicate and then incubating the plates at 37°C for 48±2 hours. Between 1 and 25 colonies were counted per spot, colonies were identified as *Salmonella* by morphology and black color indicating hydrogen sulfide formation.

Cecal content samples were also enriched in Tetrathionate Broth (TT, Hardy Diagnostics) with 2% Iodine-Iodide solution by adding 1 ml of -1 dilution mixture to 9 ml of TT in 15 ml conical vials (VWR) and then incubating at 37°C for 48 hrs. After incubation, the samples were vortexed and streaked onto XLT4+ and XLT4 into quadrants with 1 µl disposable plastic inoculation loops (VWR Scientific).

LG samples for each pen were created by collecting a total of ~150g litter from three areas of each pen. These areas were beside the feeder, under the water lines, and in between the two. Collected litter was placed into 532 ml puncture proof bags (VWR, Nasco Whirl-Pak). Each LG samples was enumerated by placing 10 grams of litter into Whirl-Pak Homogenizer Filter Bags (Nasco Whirl-Pak, Madison, Wisconsin), diluting with 90 ml of PBS and stomaching for one minute. Litter samples were enriched by adding 1 g of litter to 9 ml of TT broth with 2% Iodine-Iodide solution into 15ml conical vials and incubated at 37°C for 48hrs. After that time 10µl was streaked on XLT4 and XLT4+ in quadrants with 10 µl disposable plastic inoculation loops.

LG samples were enumerated on XLT4+ and XLT4 by spot plating 10 µl onto grid plates (VWR Scientific) in triplicate and then incubating the plates at 37°C for 48±2 hours. Between 1

and 25 colonies were counted per spot, colonies were identified as *Salmonella* by morphology and black color indicating hydrogen sulfide formation.

Statistical Analysis

All statistical analyses were conducted using SPSS Software version 26 (IBM, Armonk, NY). All cecal content and LG *Salmonella* counts were log10 transformed and analyzed by GLM with means separated by Tukey's HSD. Significant differences were reported when $P \leq 0.05$. The number of positive cecal content samples and litter grab samples between treatment groups, between sampling days within each treatment group, and between ceca enrichment were analyzed using Chi-square test or Fisher's exact test if appropriate with significant differences reported when $P \leq 0.05$.

RESULTS AND DISCUSSION

Experiment 1

The analysis of data collected during E1 revealed differences in Nalidixic acid susceptible *Salmonella* (NS) counts observed in cecal contents. Differences were observed over time within SK and SK/SE sampling groups (Table 5.2). NS counts observed in the SK group decreased from 2.77 log10 cfu/g on D14 to less than 1.00 on days 21, 28, 35, 42 and 49. Differences were observed between D14 and all other days ($P<0.001$). NS counts observed in the SK/SE group decreased over time although the only significant difference was between D14 with 1.57 log10 cfu/g versus 0.2 on D28 ($P=0.007$). Differences observed in NS colonization over time confirms that, as birds mature, *Salmonella* is shed and sometimes eliminated from their intestinal microflora.

The NS counts calculated from sampling during E1(D14-D49) were different between treatments groups (Figure 5.1). Differences were observed between SK with an average of 1.01 log10 cfu/g observed versus 0.55 in the SE/SK group ($P=0.01$) and versus 0.38 in the SE group

(P<0.001). Differences were also reported between the SK/SE group with a calculated average of 0.78 log10 cfu/g versus 0.38 in the SE group (P=0.026). The observed differences between the SK and the SE/SK group suggest that the initial *Salmonella* challenge makes it difficult for a subsequent different Salmonella serovar to colonize the ceca. These findings agree with reports from Pineda et al (2021), although this study assessed colonization of serovars over several weeks versus their study in which they made these observations over a few days. SK was introduced only two days prior to the introduction of SE, differences were still observed but could be magnified if SK were introduced on D0 versus D5 which would allow more time for the colonization. The administration of *Bacillus subtilis* spores 24h prior to SE challenge did reduce SE colonization (La Ragione & Woodward, 2003), so it is possible that the smaller window between challenging with different *Salmonella* serovars may have no effect. These findings also agree with the competitive exclusion principles established by utilizing other microorganisms to exclude SE (La Ragione & Woodward 2003; Nakamura et al., 2002; Nisbet, 2002; Zhang and Doyle, 2007; Vilà et al., 2009). Additionally, we did see that the SE group did have some counts for NS, but this is most likely due to error of the approximation that we had to use in order to calculate NS.

When analyzing results between challenge groups on sampling days, differences in NS counts were observed on D14 and D28 (Figure 5.1). NS counts were observed to be 2.77log10(cfu/g) in the SK group on D14 versus 0.47 in the SE and SE/SK groups (P<0.001) and 1.57 in the SK/SE group (P=0.045). Although over 1 log of numerical differences in counts were observed on D14 between SK/SE and the SE/SK and SE groups, no statistical difference was observed (P=0.08). The difference observed between the SK and SK/SE group may indicate that there was some competition between serovars when challenged within 48 hours of each other

although there is a possibility that SK colonized the ceca longer than SE or was better at colonizing the ceca than SE. Additionally, the differences between the SK and SE/SK group on D14 further confirms that the introduction of one serovar of *Salmonella* negatively effects the bacterial load or colonization of the subsequent serovar. Differences were also observed on D28 with 0.95log10(cfu/g) observed in the SK group versus 0.25 in the SE/SK group (P=0.025), and 0.20 in the SK/SE group (P=0.014) with the SE group being intermediate with NS counts observed to be 0.40. The remaining days showed no differences between groups.

The prevalence (+/-) of NS in cecal contents was reported in Table 5.3. Differences over time were within the SE/SK, SK, and SK/SE groups. Differences in prevalence observed in the SE/SK group were between D14 (13/25) and D28 (4/25) (P=0.007), D42 (5/25) (P=0.02), D49 (6/25) (P=0.04), and also between D21 (11/25) and D28 (4/25) (P=0.03). Differences within the SK group were between D14 (23/25) and D21 (9/25) (P<0.001), D35 (8/25) (P<0.001), D42 (11/25) (P<0.01), D49 (12/25) (P<0.001), and also between D28 (18/25) and D21 (P=0.01), D35 (P<0.01), and D42 (P=0.04). Differences within the SK/SE group were between D14 (16/25) and D28 (6/25) and D35 (7/25) (P≤0.01). These differences display similar observations of NS colonization decreasing as birds age due to shedding of *Salmonella* from their intestinal microflora.

The total proportion of samples positive for NS displayed differences when compared between treatment groups (Table 5.3). The total proportion of NS positive samples was 81/150 in the SK group which was significantly greater than 42/150 in the SE group (P<0.001), 54/150 in the SE/SK group (P<0.001), and 60/150 in the SK/SE group (P=0.015). Additional differences were observed between the SE and SK/SE groups (P=0.033). The difference observed between the SK and SE/SK contributes to the hypothesis of one serovar decreasing the colonization of the

subsequently introduced serovar which was noted in Table 3.1 and by Pineda et al. (2021). The differences in prevalence between SK and SE/SK also further suggests the concept of competitive exclusion of *Salmonella* by challenging with other microorganisms first (La Ragione & Woodward 2003; Nakamura et al., 2002; Nisbet, 2002; Zhang & Doyle 2007; Vilà et al., 2009). The approximation used for NS prevalence could explain the prevalence of NS in the SE pens. In the incident of a sample testing positive for NR and NS, the sample was considered mixed culture where in some circumstances that may not be the case. Further characterization would be necessary to differentiate in this situation.

Naldixic acid resistant *Salmonella* (NR) counts in cecal contents were found to differ over time in E1 (Table 5.4). Differences were observed between averages calculated for sampling days within SE, SE/SK, and SK/SE. NR counts within the SE group were 4.11 log10(cfu/g) on D14, 3.43 on D21, 2.50 on D28, 0.70 on D35, 0.93 on D42, and 0.32 on D49. Differences between NR counts in the SE group were between D14 and D28, D35, D42, D49, as well as D21 and D28 compared to D35, D42, D49 ($P<0.01$). NR counts within the SE/SK group were 4.45 log10(cfu/g) on D14, 2.71 on D21, 2.23 on D28, 1.11 on D35, 1.2 on D42, and 0.13 on D49. Differences within the SE/SK group were between D14 and D21. D28, D35, D42, and additionally between D21 and D35, D42, D49, with an additional difference between D28 and D49 ($P\leq0.01$). NR counts observed within the SK/SE group were 2.29log10(cfu/g) on D14, 2.58 on D21, and 2.63 on D28 which were all statistically different than 0.68 on D35, 0.77 on D42, and 0 on D49 ($P<0.001$). The reported data leads to the conclusion that NR was also shed from the cecal contents of broilers over time and that NR counts decreased similarly to decreases reported in NS counts.

The *Salmonella* count averages calculated from data collected during E1 (D14-D49) are presented in Figure 5.2. The overall *Salmonella* averages calculated for NR were 2.00 log10(cfu/g) in the SE group, 1.97 in the SE/SK group, which were statistically different than 0.18 in the SK group while the SK/SE group (1.49) was intermediate to SE (2.0) and the SK (0.18) groups (P=0.04). The differences observed between the SE and SK/SE agree with the findings of Pineda et al. (2021) and the concept of competitive exclusion reported by other groups (La Ragione & Woodward 2003; Nakamura et al., 2002; Nisbet, 2002; Zhang & Doyle 2007; Vilà et al., 2009), challenging with SK before the introduction of SE reduced the bacterial load of SE. Additionally, the similarity between NR bacterial load of cecal contents between SE and SE/SK implies that the addition of SK one week after the initial challenge of SE at D0 had no effect on SE bacterial load.

The comparison of NR between treatment groups within sampling days displayed differences between challenge groups is presented in Figure 5.2. NR average counts calculated for D14 were 4.11 log 10(cfu/g) in the SE group and 4.45 in the SE/SK group which were statistically different than 0.17 in the SK group, and 2.29 in the SK/SE group with SK/SE being intermediate to SE, SE./SK and SK (P<0.001). The differences observed on D14 further confirm that the introduction of SK before SE significantly affected the bacterial load of SE. However, the introduction of SK after challenging with SE had no effect on SE bacterial load. This finding was different than the differences seen in NS counts observed in E1 (Figure 3.1), which showed the bacterial load of SK to be affected negatively by the subsequent introduction of SE. This effect was most likely not observed in SE bacterial load of the SE/SK group due to SE challenges being performed on D0 versus the primary SK challenge on D5, therefore allowing SE more time to colonize the ceca without competition. The difference may also be due to SK

not colonizing as readily due to challenging on D5 versus D0, which would agree with the findings that younger chicks are more readily colonized by *Salmonella* due to their lack of intestinal microflora (Milner & Shafer, 1952). Additional differences were observed between SK and all other groups on D14, D21, D28, and D35 ($P \leq 0.032$). On D42 SE/SK (1.2) had statistically higher NR levels than SK (0.13) ($P<0.01$). SE (0.93) and SK/SE (0.77) were intermediate compared to SE/SK and SK. No differences were observed between groups on D49. Although differences were observed between co-challenged groups on Day 14, no differences were observed between these groups in the subsequent weeks. Cecal contents of birds at market age (35-49 days) contained the same bacterial load of NR *Salmonella* regardless of treatment.

The prevalence of NR in cecal contents was observed to have differences between sampling days within each challenge treatment group (Table 5.5). The proportion of positive samples in the SE group were 24/25 on D14, 22/25 on D21, 23/25 on D28, 11/25 on D35, 16/25 on D42, and 5/25 on D49. Differences observed within the SE treatment group were between Days 14, 21, 28, 42 and Days 49 and between Day 35 and 49($P<0.047$). NR prevalence in the SE/SK group was 25/25 on D14, 20/25 on D21, 22/25 on D28, 18/25 on D35, 16/25 on D42, and 1/25 on D49. Differences were observed between D14 and D35, D42, and D49 ($P<0.01$) and between D28 and Day 28 and Days 42 and 49 ($P<0.047$). In the SK group, NR prevalence was observed to be 1/25 on D14, 10/25 on D21, 5/25 on D28, 0/25 on D35, 1/25 on D42, and 0/25 on D49 with differences observed between D21 and Days 14, 35, 42, and 49 ($P<0.01$). The proportions of NR positive SK/SE samples were 16/25 on D14, 20/25 on D21, 23/25 on D28, 15/25 on D35, 9/25 on D42, and 0/25 on D49. Statistical differences observed within the SK/SE challenge group were between D28 and Days 14, 35, 42, and 49 ($P<0.02$). Other differences were observed on D14 compared to Days 42 and 49 ($P<0.002$). Day 49 had the lowest NR

prevalence in the SK/SE challenge group compared to all other days (P<0.002). Prevalence of NR *Salmonella* decreased over time in all groups which was similar to the observed decreases in NS *Salmonella* counts. It is noteworthy that NR prevalence did decrease to 0 at D49, this observation may indicate that the challenge of SK before the introduction of SE may have negatively affected the ability of SE to persist in the ceca over time.

The total prevalence of NR during E1 (D14-D49) showed some differences between groups. (Table 5.5). The prevalence of NR (SE) in all collected cecal content was 101/150 for SE and 102/150 for SE/SK which was statistically higher than 17/150 for SK and 83/150 in SK/SE (P<0.03). SK had the lowest overall prevalence while SK/SE was between the two extremes. The differences observed between SE and SK/SE and between SE/SK and SK/SE further implicate an exclusion effect on SE by previously introduced SK. Although SK was only introduced 2 days before SE, colonization of the ceca by SE was still reduced. There was some NR present in SK group, this could be due to nalidixic acid resistant *Salmonella* spp. remaining in the environment from previous trials or potential cross contamination at necropsy. Full clean outs are done in this facility so NR remaining in the environments from previous trials is unlikely.

Table 5.6 summarizes data observed from LG sampling litter on D49. No differences were observed in NS counts between groups although numerical differences were observed with 2.66log10(cfu/g) observed in the SE group, 0.78 in SE/SK, 0.20 in SK, and 1.57 in SK. However, differences NS prevalence were observed between SE (4/5) and SK (1/5) (P=0.03) while the other two challenge groups were not significantly different. No differences were observed between NR recovery from the litter in NR counts or prevalence. No *Salmonella* was recovered from the litter on D63 (data not shown). This data suggests that challenging with

different serovars may not correlate with what can be recovered from the litter or that survival in the environment may differ between serovars.

Experiment 2

The prevalence of NS in cecal contents was very low in the subsequent flock after placing chicks on the same litter (E2) (Table 5.7) and no NR *Salmonella* was recovered from the cecal contents of any birds during E2 (data not shown). There were no differences in NS prevalence observed over time, however, differences were observed between treatment groups in the total proportion of samples positive for NS. Overall prevalence of NS was 3/150 in SE, 11/150 in SE/SK, 0/150 in SK, and 6/150 in SK/SE. Differences were observed between SE/SK and SE and SK ($P<0.029$); as well as between SK/SE and SK ($P=0.03$). It was observed that NS persisted in the environment at adequate levels to infect birds of the subsequent flock, especially in the groups that were co-challenged with SK on D7. It is noteworthy that not all NS samples can be confirmed as the original SK isolate, although C_2 *Salmonellae* were confirmed during E2, some group D_1 isolates were confirmed during E1 that were susceptible to Nalidixic acid and additional SK introduced from other sources could not be distinguished from the SK isolate that was administered to birds. The detection of other *Salmonella* serovars is possible because not all samples were characterized by serogroup. Additionally, it is interesting that the birds raised in the same pens as the co-challenged groups were found to have more positive cecal content samples versus the SK group. Only one pen was found to be positive for NS *Salmonella* when sampling pens with LG on D49, which was in the SK/SE group (data not shown). This data suggests that the SK isolate utilized in this experiment persisted in the environment longer than the SE isolate. However, it is possible that the *Salmonella* isolated from this litter sample came from the hatchery or was introduced into the environment from another source, although this

isolate was identified in the C_2 serogroup. With SK being the most frequently isolated serovar from poultry products (CDC, 2016), the observations of this study confirm its resiliency in the environment.

CONCLUSION AND APPLICATIONS

- Challenging with SE before SK (SE/SK) negatively affected the colonization NS *Salmonella* in cecal contents
- Challenging with SK before SE (SK/SE) negatively affected the bacterial load of NR *Salmonella* in cecal contents during E1 and on D14
- The colonization of NS *Salmonella* of cecal contents was higher in co-challenged groups (SK/SE and SE/SK) than SK during E2.
- The initially introduced serovar took precedence over the subsequently introduced serovar in colonization and bacterial load of cecal contents

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DATA

Table 5.1: E1 *Salmonella* Challenge Schedule by Treatment

Treatment/Day:	SE	SE/SK	SK	SK/SE
D1	10^7 SE	10^7 SE	N/A	N/A
D5	N/A	N/A	10^7SK	10^7SK
D7	N/A	10^7SK	N/A	10^7 SE

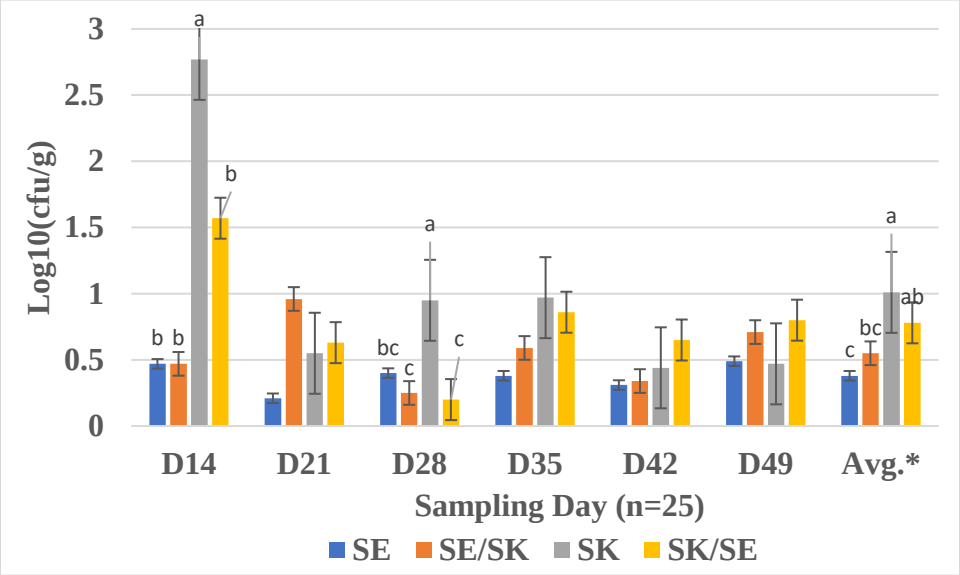
Description: This table depicts the days and dosages of the *Salmonella* challenges of each serovar for each treatment group.

Table 5.2: E1 Cecal Content NS Counts

(n=25)	SE	SE/SK	SK	SK/SE
D14	0.47	0.47	2.77^{a}	1.57^{a}
D21	0.21	0.96	0.55^{b}	0.63^{ab}
D28	0.40	0.25	0.95^{b}	0.20^{b}
D35	0.38	0.59	0.97^{b}	0.86^{ab}
D42	0.31	0.34	0.44^{b}	0.65^{ab}
D49	0.49	0.71	0.47^{b}	0.80^{ab}

Description: This table depicts the average bacterial load of nalidixic acid susceptible *Salmonella* in cecal contents of birds during each sampling day from D14-D49 during E1. Differences in *Salmonella* load between sampling days within each group are displayed by $^{a\text{-}b}$ values within columns, with differing letters showing statistical differences ($P \leq 0.05$).

Figure 5.1: E1 Cecal Content NS Counts



Description: This figure depicts the average bacterial load of nalidixic acid susceptible *Salmonella* in cecal contents of birds during each sampling day from D14-D49 of E1 with a group on the far right for the total calculated average bacterial load for each group (D14-D49). Differences in *Salmonella* load between groups within each sampling day are displayed by $^{a\text{-}c}$ values within columns, with differing letters showing statistical differences ($P \leq 0.05$).

Table 5.3: E1 Cecal Content NS Prevalence

	SE	SE/SK	SK	SK/SE
D14	10/25	13/25[1]	23/25[1]	16/25[1]
D21	9/25	11/25[1,2]	9/25[3]	10/25[1,2]
D28	4/25	4/25[3]	18/25[1,2]	6/25[2]
D35	7/25	8/25[1,2,3]	8/25[3]	7/25[2]
D42	5/25	5/25[2,3]	11/25[3]	10/25[1,2]
D49	6/25	6/25[2,3]	12/25[2,3]	11/25[1,2]
Total	42/150[c]	54/150[bc]	81/150[a]	60/150[b]

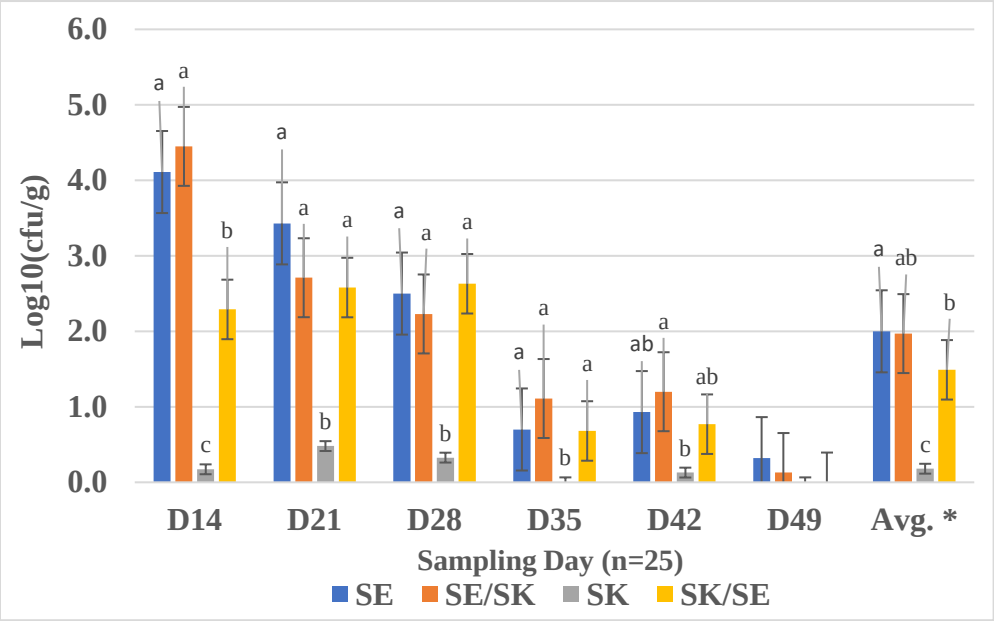
Description: This table depicts the prevalence of nalidixic acid susceptible *Salmonella* in cecal contents of birds during each sampling day from D14-D49 during E1. Differences in prevalence between sampling days within each group are displayed by [1-3] values within columns, with differing letters showing statistical differences ($P \leq 0.05$). Differences between the total prevalence from D14-D49 are displayed by [a-b] values within the bottom row with differing letters showing statistical differences ($P \leq 0.05$)

Table 5.4: E1 Cecal Content NR Counts

(n=25)	SE	SE/SK	SK	SK/SE
D14	4.11[a]	4.45[a]	0.17	2.29[a]
D21	3.43[ab]	2.71[b]	0.48	2.58[a]
D28	2.50[b]	2.23[bc]	0.33	2.63[a]
D35	0.70[c]	1.11[cd]	0	0.68[b]
D42	0.93[c]	1.20[cd]	0.13	0.77[b]
D49	0.32[c]	0.13[d]	0	0[2]

Description: This table depicts the average bacterial load of nalidixic acid resistant *Salmonella* in cecal contents of birds during each sampling day from D14-D49 during E1. Differences in *Salmonella* load between sampling days within each group are displayed by [a-d] values within columns, with differing letters showing statistical differences ($P \leq 0.05$).

Figure 5.2: E1 Cecal Content NR Counts



Description: This figure depicts the average bacterial load of nalidixic acid resistant *Salmonella* in cecal contents of birds during each sampling day from D14-D49 of E1 with a group on the far right for the total calculated average bacterial load for each group (D14-D49). Differences in bacterial load between groups within each sampling day are displayed by $^{a\text{-}c}$ values within columns, with differing letters showing statistical differences ($P \leq 0.05$).

Table 5.5: E1 Cecal Content NR Prevalence

	SE	SE/SK	SK	SK/SE
D14	24/25[1]	25/25[1]	1/25[2]	16/25[2]
D21	22/25[1]	20/25[1,2,3]	10/25[1]	20/25[1,2]
D28	23/25[1]	22/25[1,2]	5/25[1,2]	23/25[1]
D35	11/25[2,3]	18/25[2,3]	0/25[2]	15/25[2,3]
D42	16/25[2]	16/25[3]	1/25[2]	9/25[3]
D49	5/25[3]	1/25[4]	0/25[2]	0/25[4]
Total	101/150[a]	102/150[a]	17/150[c]	83/150[b]

Description: This table depicts the prevalence of nalidixic acid resistant *Salmonella* in cecal contents of birds during each sampling day from D14-D49 during E1. Differences in prevalence between sampling days within each group are displayed by [1-4] values within columns, with differing letters showing statistical differences ($P \leq 0.05$). Differences between the total prevalence from D14-D49 are displayed by [a-c] values within the bottom row with differing letters showing statistical differences ($P \leq 0.05$).

Table 5.6: E1 D49 Litter Grab

	SE	SE/SK	SK	SK/SE
NS Counts	2.66	0.78	0.20	1.57
NS	4/5[a]	2/5[ab]	1/5[b]	3/5[ab]
NR	0/5	3/5	0/5	1/5

Description: This table depicts the litter grab NS and NR counts and prevalence observed on D49 of E1. Differences within the NS group prevalence are shown by [a-b] values with differing letters showing statistical differences ($P \leq 0.05$).

Table 5.7: E2 Cecal Content NS Prevalence

	SE	SE/SK	SK	SK/SE
D14	0/25	2/25	0/25	0/25
D21	0/25	2/25	0/25	0/25
D28	0/25	2/25	0/25	0/25
D35	2/25	2/25	0/25	2/25
D42	0/25	2/25	0/25	0/25
D49	1/25	1/25	0/25	4/25
Total	$3/150^{bc}$	$11/150^{a}$	$0/150^{c}$	$6/150^{ab}$

Description: This table depicts the prevalence of nalidixic acid susceptible *Salmonella* in cecal contents of birds during each sampling day from D14-D49 during E1. Differences between the total prevalence from D14-D49 are displayed by $^{a\text{-}c}$ values within the bottom row with differing letters showing statistical differences ($P \leq 0.05$).

Chapter 6:

Conclusion

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Conclusion

Poultry meat is now the most consumed meat per capita in the United States (Kuck and Schnitkey, 2021), which makes efforts to control Nontyphoidal *Salmonella* important in protecting consumers from Salmonellosis, which results in a large proportion of deaths and illnesses among foodborne illnesses (CDC, 2019). Advancements in *Salmonella* spp. detection methods, management techniques, and the understanding of how *Salmonella* spp. colonizes poultry and spreads throughout areas of poultry production are important parts of controlling this bacterium. Although Nontyphoidal *Salmonella* is endemic in environments housing poultry, methods to mitigate contamination and spread of *Salmonella* spp. is still worth the efforts of food safety experts and scientists all over the world.

The objective of Chapter 3.0 was to compare Roller Swabs to other litter sampling methodology and to further refine these methods so that *Salmonella* spp. can be detected in the environment efficiently and practically. In Experiment 1, Roller Swabs were not as sensitive other methods in an environment where *Salmonella* spp. was highly prevalent ($P<0.001$). However, in lower prevalence, Roller Swabs were intermediate in detection between Drag Swabs, which were the lowest in detection, and Boot Covers ($P\leq0.022$). Boot covers were the highest in sensitivity, which is in agreeance with other groups (Caldwell et al., 1998; Skov et al., 1999; McCrea et al., 2005; Buhr et al., 2007). Additionally, Roller Swabs were more practical than other methods in sampling research pens due to the ability to sample pens without entering pens. This makes efforts to reduce cross contamination between pens easier while sampling the litter. With some refinement, this method could be helpful in research environments to assess *Salmonella* spp. prevalence.

Chapter 4 aimed to compare five litter treatments by their effect on *Salmonella* Enteritidis prevalence and transmission of *Salmonella* Enteritidis (SE) from one flock to chicks raised on the same litter. Although some differences were seen in Litter Grab with Sodium Bisulfate having higher prevalence than Sodium Formate Salts, Untreated Control, and Windrow Composted pens ($P<0.05$); no differences in SE counts by Litter Grab or in prevalence detected by Litter Grab or Boot Covers at any other time. None of these treatments had any significant effect on the prevalence of SE or on the transmission of SE from the seeder flock to the flock raised in the same environment. Sodium Formate salts did slightly reduce aerobic bacteria, *Escherichia coli*, and total coliform counts after reusing the litter three times ($P<0.05$). Reductions in aerobic bacteria could potentially be increased by using a higher application rate, Macklin and Krehling saw higher reductions when using higher application rates of metam sodium (Macklin & Krehling, 2010). Additionally, Windrow Composting showed similar reduction in *E. coli* and total coliform counts, but further reduction could have been possible by turning piles to ensure even heating of the litter. Further investigation of Sodium Formate Salts and Windrow Composting while turning is needed to confirm if more significant reductions in bacterial counts can be achieved.

The discovery of non-marker strain *Salmonella* spp. in the ceca of market age birds in Experiment 3 of Chapter 4 lead to the question if one serovar of *Salmonella* spp. could exclude the colonization of a subsequently introduced serovar in the ceca of broilers. Differences in nalidixic acid susceptible *Salmonella* counts and prevalence, which aimed to quantify *Salmonella* Kentucky, were observed between SK and SE/SK groups ($P<0.01$). The use of a *Salmonella* Kentucky marker strain would have helped legitimize *Salmonella* Kentucky counts (NS) and prevalence, although these finding suggest some competition between serovars. Additionally,

differences in nalidixic acid susceptible counts were observed between SE and SK/SE ($P<0.05$). Differences in prevalence were also observed between not only SE and SK/SE, but also between SE/SK and SK/SE ($P<0.05$). These results suggest a stronger argument that SK did reduce the colonization of SE due to SE being the marker strain resistant to nalidixic acid. Pineda et al (2021) reported similar results when challenging with two different marker strains of *Salmonella* spp. in chicks that were 1 and 2 days of age. The concept of competitive exclusion of *Salmonella* spp. in the gastrointestinal tract of chickens suggests the possibility of developing a *Salmonella* surrogate or similar product to exclude or reduce *Salmonella* spp. colonizatiions, which could be a notable advancement in *Salmonella* spp. control.

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