EVALUATING THE ADDITION OF CHARCOALS TO BROILER DIETS ON THE RECOVERY OF *SALMONELLA* TYPHIMURIUM DURING GROW-OUT AND PROCESSING

by

KIMBERLY MARIE WILSON

(Under the Direction of R. Jeff Buhr)

ABSTRACT

Two experiments evaluated charcoals added to the feed on the recovery of *Salmonella* in broilers during grow-out and processing. In both experiments, 2 "seeder" chicks were inoculated with *Salmonella* Typhimurium and placed with penmates to spread Salmonella throughout the pen. Feeding treatments for experiment 1 were: basal control, 0.3% bamboo charcoal, 0.6% bamboo charcoal or Aromabiotic® (8 pens/TRT). The ceca penmates were sampled to confirm *Salmonella* colonization at 3, 4 and 6 wk, and pen litter was sampled weekly. At 3 wk, charcoal fed chicks had significantly lower recovery of *Salmonella* via direct plating. At 6 wk, Aromabiotic had significantly lower recovery of *Salmonella* with enrichment. In experiment 2 the treatments were: basal control; 0.3% bamboo charcoal; 0.3% activated bamboo charcoal or 0.3% pine charcoal (10 pens/TRT). Penmate ceca were sampled at 1 and 2 wk (1 penmate/pen) and 5 penmates/pen at 3 to 6 wk. The pH of the crop and duodenum was measured weekly from 1 penmate/pen, and litter was sampled weekly. At the end of grow-out, broilers were processed at two consecutive days. Results showed that penmates were colonized at 1 wk. Cecal *Salmonella* showed no difference except at 4 wk, activated bamboo charcoal had a 14% lower

recovery of *Salmonella* with enrichment compared to the control. Recovery of *Salmonella* from the litter was not different among treatments, showing similar trends as in experiment 1, an overall decrease in recovery by 4 wk with direct plating. The pH of the duodenum and the crop were not different among treatments. Crop pHs from all treatments were significantly higher at wk 1 compared to wk 2 thru 6. Although litter recovery of *Salmonella* was not significant among treatments, charcoal had minimal effect on the recovery in the ceca, a significant reduction on the recovery from breast skin (20% decrease) was observed.

INDEX WORDS: *Salmonella*, Broilers, Charcoal, Ceca

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DEDICATION

I would like to dedicate my work to my mom, Patty Wilson. Thank you for being my number 1 cheerleader and supporter throughout my academic career. You have always been the woman I can count on for anything, and I hope to always make you proud.

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CHAPTER 1

INTRODUCTION

The contamination of broiler carcasses with *Salmonella* remains a tremendous problem and concern for the poultry industry and regulatory agencies. The process of growing broiler chickens gives rise for opportunities of pathogens such as *Salmonella* to be transferred from the environment to the chick and then from bird to bird. Cox and coauthors suggested that the newly hatched chick may be exposed to significant levels of *Salmonella* from an assortment of sources such as the hatching cabinet, hatchery environment, and the broiler house (Cox et al. 1996). Once *Salmonella* reaches the primary site of colonization, the ceca of a young chick (Milner and Shaffer 1952, Brownell et al 1969, Barrow et al., 1988, Fanelli et al 1971, Snoeyenbos et al 1982), and it is speculated that *Salmonella* may attach to the intestinal mucosa to colonize (Barrow et al. 1988). By the time broilers reach the market age and weight, *Salmonella* may be recovered at lower numbers from the crop or ceca but can remain on the carcass and be recovered after processing on the final product.

Prebiotics added to feed are considered to be a possible answer to contribute to lowering the recovery of *Salmonella* without the use of antibiotics, which are not allowed in the European Union (European Commission, 2011). Prebiotics may work by either binding to the bacterial pathogen in the intestinal lumen and blocking the adhesion of bacteria to the epithelia cell surface (Spring et al., 2000), or prebiotics may provide a substrate for the metabolism and growth of intestinal flora, thus inhibiting pathogen colonization by competitive exclusion (Suskovic et al., 2001). Charcoal is a carbanecaous material that has high porosity and immense

internal surface area. Charcoal generally refers to the carbonaceous residue of wood; cellulose, coconut shells or other various industrial wastes left after heating organic matter in absence of oxygen. Adsorbance by activated charcoal depends on pore size and surface area, concentration, and chemical nature of the source of the charcoal (Diamadopoulos et. al., 1992).

The theory is that *Salmonella* being the ideal size, 2 by 0.5 µm, may be one of the causations for activated charcoal to readily adsorb *Salmonella*. Charcoal alone has not been tested or reported for adsorbance of *Salmonella* in a large scale in-vivo in poultry during production.

The objective of these studies was to first evaluate the colonization and recovery of *Salmonella* Typhimurium using different concentrations of bamboo charcoal added to broiler feeds. The second experiment explored the use of different types of charcoal, pine charcoal or bamboo charcoal (activated and non-activated) on the colonization and recovery of *Salmonella*, their effects on pH of the crop and duodenum, and at the end of grow-out the impact on residual *Salmonella* on the breast skin of the carcasses post defeathering. Finally, a performance study was conducted to confirm if there was an effect on body weight and feed to gain ratio with the addition of bamboo charcoal and pine charcoal at the same ratios of the second experiment.

CHAPTER 2

LITERATURE REVIEW

Overview on history of *Salmonella*

Salmonella are gram-negative non-spore forming bacteria that belong to the *Enterobacteriaceae* family. *Salmonella* comprises of 2 species *S. bongori and S. enterica*. Within the *Salmonella enterica* species, there are 6 subspecies, *enterica, salamae, arizonae, diarizonae, houtenae* and i*ndica* (Coburn et al., 2007). The subspecies *enterica* is commonly associated in food-borne *Salmonella*. Within the subspecies of *enterica*, there are over 1500 different serotypes, including Typhimurium, Enteritidis, Heildberg, Kentucky which are a few serotypes that are commonly found in poultry. *Salmonella enterica* subspecies can be characterized immunologically on the basis of cell surface antigens, the O or cell wall (somatic) antigen, the H antigen for the flagella, and the capsular Vi antigens. The most common O-antigen serogroup within *S. enterica* subspecies are A, B, C1, C2, D, and E strains (Brenner et al. 2000). For example, *Salmonella enterica* serovar Typhimurium, commonly referred as *Salmonella* Typhimurium, is in serogroup B while *Salmonella* Enteritidis is in serogroup D. The number of serovars in the *enterica* subspecies is approximately 1427 (Lund et al 2000). Of the more than 2,500 *Salmonella* serotypes, only 10% is associated with the commercial egg and poultry industry (Gast 2007, Lutful Kabir 2010).

Salmonella was originally discovered by technician Theobald Smith in 1885, though Daniel Salmon, research leader and veterinarian had *Salmonella* named after himself (Salmon and Smith 1884-1886). The *Bacterium enteritidis* was the first isolated food-poisoning

Salmonella in 1888 in Germany. It was cultured from a slaughtered cow and caused 58 cases of food poisoning, including one man who died 36 h after consuming 1 ½ lbs of meat. At this time, the poultry industry was poorly developed, red meat was then the most important source of these organisms (Barrow 1993).

During the early 1900's, *Salmonella enterica* serovars Pullorum (common name pullorum disease) and Gallinarum (common name fowl typhoid) were a wide spread poultry disease problem in the United States (Shivaprasad 2003). The National Poultry Improvement Plan was established in 1935 in order to control the economic losses caused by these poultry pathogenic bacteria (USDA 1997). *Salmonella* Pullorum and Gallinarum outbreaks reduced considerably by the mid 1960's in the United States (Bäumler et al. 2000) and in other countries going from separate businesses of poultry producing to the current intensive and integrated poultry industries, large-scale incubation methods, mass transportation of day-old chicks (Barrow 1993) and in the 1950's a vaccine created for *S*. Gallinarium (Smith 1956). The interest over the past few decades has switched largely to concerns over control of food poisoning *Salmonellae.*

Salmonellosis is the gastrointestinal disease typically caused by foodborne *Salmonella* infection. Human *salmonellosis* is typically associated with the consumption of contaminated foods, such as fresh and processed meat, eggs, and produce (Borland, 1975; Holmberg et. al 1984; Branham et al 2005; Benenson et al. 1995, Mead et al. 1999, Tauxe 1991). Meat and poultry consumption has been on the rise in the United States, with the per capita consumption of poultry products increasing 6.5-fold since 1910 (Buzby et al. 2006).

Salmonella enterica infections cause a significant public health concern worldwide, with an estimated 1.03 million cases, 19,000 hospitalizations, and approximately 400 deaths in the United States each year (Scallan et al. 2011). Fresh and processed poultry account for

approximately 29% of all *Salmonella* infections in humans (Braden 2006). The most commonly identified serovars associated with human infections in the United States are *Salmonella enterica* serovars Typhimurium, Enteritidis, Newport, and Heidelberg (CDC 2008). In recent years, *S.* Heidelberg and *S.* Kentucky are two serovars that have been frequently isolated from pre and postharvest poultry sources (Fricke et al. 2009, Liljebjelke et. al 2005, Nayak et. al 2008).

Salmonella colonizes poultry through fecal-oral transmission (Foley et al. 2008, Lutful Kabir 2010) however, in newly hatched chicks, colonization can also take place through the nasal cavity or cloaca and lead to the gut (Brito et al. 1995, Fedorka-Cray et al. 1995). *Salmonella* can then be spread quickly to non-contaminated chickens through contact, as well as through the common fecal-oral route. Only a small percentage of chicks need to be positive with Salmonella to infect the entire flock. A study showed, 5% of chicks were positive with *Salmonella* Typhimurium when entering the growing house and in 3 wks, *Salmonella* spread up to 95% of the flock (Byrd et al., 1998).

S. Enteritidis infection was associated primarily with rodents (Calloway et al. 2008). In addition, mathematical models have suggested that *S.* Gallinarum competitively excluded *S.* Enteritidis in poultry (Rabsch et al. 2000). Harvey and Prince 1967 found that inoculated crushed bones plated at different subculture times from the same enrichment broth showed that 6 *Salmonella* serotypes, *S.* Derby, Enteritidis, Newport, Oranienburg, Reading and Typhimurium competed with one another. *S.* Enteritidis for example 27% were found at 18 h post inoculation however it was not found at 24 h while 77% of colonies picked were *S.* Typhimurium.

Commercial poultry is one of the fastest growing sectors of the animal agricultural industry (Herren 2000). In 2006, commercial poultry management systems represented 95% of poultry production in the United States (MacDonald 2008). The majority of broilers in the United States are reared in large housing operations consisting of 6,000 to 40,000 birds per housing unit (Herren 2000). Broilers are generally raised cage-free in barns on litter, with the stocking density ranging from 6.5 to 8.5 lb/ft^2 (0.27 to 0.36 kg/m^2) depending on the size of the birds (National Chicken Council 2010).

Prebiotics

Prebiotics are feed ingredients that are non-digestible that interact with the flora of the alimentary tract. Prebiotics in diets are beneficial to the host by stimulating the growth of one or more bacteria in the alimentary tract (Gibson et al. 1995, Cummings et al 2001). In the lower intestine, prebiotics serve selectively as a substrate to stimulate the growth or activity of bacteria in the gastro-intestinal tract that are beneficial to the host health (Grizard et al. 1999). Common prebiotics are dietary fibers. Prebiotics that are considered dietary fibers are predominantly a constituent of plant cell walls and also consist of non-starch polysaccharides (NSPs) along with non-carbohydrate compounds including lignin, protein, fatty acid, and wax (Knudsen 2001). Upon ingestion, dietary fiber may influence the GI tract by altering its microbial activities, rate of passage, metabolites, and digestive efficacy (Knudsen 2001, Wenk 2001). Polysaccharides have been identified for their potential to be utilized as prebiotics (Griggs et al. 2005, Cummings et al. 2002), possibly by reducing pH and increasing VFA concentrations (Nurmi et al. 1973, Durant et al. 2000 and Bedford 2000). Beneficial species, *Lactobacilli* and *Bifidobacteria*, are considered to be inhibitory towards pathogens such as *Salmonella* are known to be supported by some of these dietary fiber

compounds (Gibson et al 1995). The more extensively studied prebiotics sources are fructooligosaccarides (FOS), oligofructose and inulin (Macfarlane et al. 2008, Patterson et al. 2003). FOS are naturally occurring oligosaccharides that originate from plants such as onions, wheat, barley, and rye and consist of one to three fructose residues attached to a sucrose molecule.

When fed to animals, FOS have been shown to impact bacterial populations by promoting the growth of *Lactobacillus* (Mitsuoka et al. 1987) and *Bifidobacterium* spp. (Xu et al. 2003). Bailey et al. 1991 demonstrated reduced susceptibility of broilers to *Salmonella* invasion after inclusion of FOS in their diets which was explained by a probable shift in gut microorganisms. The efficiency of FOS in the same study was enhanced by a combination with a protective competitive exclusion (CE) culture which resulted in a 37% reduction of *S.* Typhimurium cecal colonization in broiler chicks 7 days post challenge of 10^6 or 10^9 colony forming units (cfu) compared to chickens given CE alone.

Lactobacillus paracasei administered in combination with FOS resulted in a significant increase of *Lactobacillus* spp., *Bifidobacterium* spp., total anaerobes, and total aerobes, as well as a decrease in *Clostridium* and *Enterobacterium* observed in piglets (Bomba et al. 2002). In a series of *in vitro* studies, (Donalson et al. 2007, Donalson et al. 2008) demonstrated that a combination of FOS, alfalfa and grain, incubated with cecal inoculum exhibited a significant reduction in *Salmonella* population, while increasing propionate, butyrate, other SCFA, and lactate. However, the *in vivo* studies with laying hens were less conclusive (Donalson et al. 2008). In at least half of the trials, the *S.* Enteritidis colonization of ovary and liver of hens fed FOS (0.375% and 0.750%) containing diets were reduced compared to hens subjected to complete removal of feed. Significant decreases in cecal *S.* Enteritidis counts were also observed

in only half of the trials. However, no substantial differences in *Salmonella* colonization of hens' organs were observed due to FOS. Although the addition of FOS to cereal or high-fiber diets did not improve the production of the total cecal VFA, hens fed high fiber with or without FOS yielded greater cecal lactic acid concentrations than hens subjected to complete removal of feed (Donalson et al. 2008).

Organic acids as a prebiotic

Organic acids are considered to be any organic carboxylic acid, including fatty acids and amino acids, of the general structure R-COOH. Organic acids primarily include the saturated straight-chain monocarboxylic acids and their respective derivatives (unsaturated, hydroxylic, phenolic, and multicarboxylic versions) (Cherrington et al., 1991). They are often generically referred to as fatty acids, short chain fatty acids, volatile fatty acids, weak or carboxylic acids (Cherrington et al. 1991) Animals do not produce short chain fatty acids (SCFA) directly. SCFAs are the major end products of carbohydrate (dietary fiber) fermentation in the colon by commensal bacteria. Diet and gut bacteria affect the amount and ratios of SCFAs, but generally acetate is most prevalent, followed by propionate and butyrate. Short-chain fatty acids (SCFA), acetate, propionate, and butyrate, are produced in millimolar quantities in the gastrointestinal tracts of food animals and humans and characteristically occur in high concentrations in regions where strictly anaerobic microflora are predominant (Acuff et al., 1987; Cherrington et al., 1991; Dickson, 1992; Hardin et al., 1995; Dorsa, 1997).

Organic acids have been utilized as food/feed additives and preservatives for preventing food deterioration. Organic acid compounds targets bacteria specifically the cell wall, cytoplasmic membrane, and specific metabolic functions in the cytoplasm associated with

replication, protein synthesis, and function (Denyer and Stewart, 1998; Davidson, 2001). The antibacterial mechanism(s) for organic acids are not fully understood, they are capable of exhibiting bacteriostatic and bactericidal properties depending on the physiological status of the organism and the physicochemical characteristics of the external environment. Given the weak acid nature of most of these compounds, pH is considered a primary determinant of effectiveness because it affects the concentration of undissociated acid formed (Davidson, 2001). It has been traditionally assumed that undissociated forms of organic acids can easily penetrate the lipid membrane of the bacterial cell and once internalized into the neutral pH of the cell cytoplasm dissociate into anions and protons (Eklund, 1983, 1985; Salmond et al., 1984; Cherrington et al., 1990, 1991; Davidson, 2001).

Organic acids also have been incorporated as spray sanitizers during poultry and other livestock processing (Acuff et al., 1987; Cherrington et al., 1991; Dickson, 1992; Hardin et al., 1995; Dorsa, 1997). In the food animal industry, organic acids were originally added to animal feeds to serve as fungistats (Paster, 1979; Dixon and Hamilton, 1981), but in the past 30 years, formic and propionic acids and various combinations have also been examined for potential bactericidal activity in feeds and feed ingredients contaminated with foodborne pathogens, particularly *Salmonella* (Khan and Katamay, 1969; Hinton and Linton, 1988, Humphrey and Lanning, 1988; Izat et al., 1990; McHan and Shotts, 1999; Berchieri and Barrow, 1996; Thompson and Hinton, 1997).

Feed and drinking water sanitation, and the addition of acid to the crop, appears to prevent pathogen colonization in the live animals, but factors and types of acids such as the chain length, pKa values, and hydrophobicity can affect its antimicrobial activity (Hsiano et al. 1999, Van Immerseel et al., 2007). *Salmonella* colonization of the ceca and internal organs is not

always affected by these treatments, especially if the level or prevalence of *Salmonella* is high or if birds are challenged with a high level of *Salmonella* (at least 10^6cfu) (Hume et al. 1993, Izat et al. 1990). Acids from feed or drinking water are not effective further down in the intestinal tract because *Salmonella* colonization is mainly in the ceca (Desmidt et al., 1997). Because the ceca is the main fermentation site, the concentrations of SCFA are already higher there than in other intestinal segments (Engberg et al., 2002). Day of hatch broilers do not have negligible acetate in their ceca and by 15 days can have up to 70 μmol g^{-1} which remains stable through grow-out (Van Der Wielen et al., 2000).

Acetic acid is the predominant short-chain fatty acid in the ceca, with concentrations ranging between 70 and 90 mmol/g cecal content (Engberg et al. , 2002; Van Der Wielen et al. , 2002). In most studies, the cecal butyrate concentration ranges between 10 and 40 mmol/g in chicken ceca, and the propionate concentration is even less (Engberg et al., 2002; Van Der Wielen et al., 2002). All of these acids start out much lower in concentration before and increase significantly and stabilizing by 15 days post hatch (Van Der Wielen et al 2000). Because SCFA can affect invasion and virulence gene expression of Salmonella (Lawhon et al. 2002; Van Immerseel et al. 2004; Gantois et al. 2006), the natural quantities of the SCFA could play an important role in *Salmonella* colonization. If SCFA production in the ceca could be altered by changes in feed composition, producers would have a very cost-effective and efficient way of controlling *Salmonella.*

Salmonella colonization of the gut from many animal species is decreased when the *bifidobacterial* population is increased, either by administration of bifidobacteria as probiotic strains or by addition of certain types of oligosaccharides that stimulation proliferation of these bacteria in the gut (Asahara et al. 2001; Buddington et al. , 2002; Bovee- Oudenhoven et al.,

2003; Silva et al., 2004; Thitaram et al., 2005). When the cecal bifidobacterium population in broilers was increased by isomalto-oligosaccharide addition to the feed, and the animals were infected with a high dose of S. Typhimurium, large reductions in cecal colonization were observed (Thitaram et al., 2005).

Lactic acid bacteria, such as *lactobacilli* and *bifidobacteria*, stimulate proliferation of butyric-acid-producing bacteria. Increases in lactic acid bacterial counts in the gut are correlated with increases in butyric acid concentrations (Kleessen et al. 2001; Humblot et al. 2005), and *Salmonella* colonization is decreased when butyric acid concentrations in the gut are increased (Van Immerseel et al., 2004, 2005). *Bifidobacteria* increase butyric acid concentrations, but these bacteria do not produce butyric acid themselves. It has been shown that lactic acid, produced *in-vitro* by *Bifidobacterium* adolescentis with starch as the sole carbon source, is used by *Anaerostipes caccae* and *Eubacterium hallii* (in co-culture) for the production of large concentrations of butyric acid (Duncan et al., 2004).

Dietary strategies to limit *Salmonella* in the avian GI tract

Recent research has shown that medium chain fatty acids (MCFA) and organic acids (SCFA) have an effect on lowering the recovery of *Salmonella* in the ceca of broilers when added to the feed and water during grow-out. In unpublished data, 2 seeders were challenged with approximately 2x 10^6 cells of *S.* Typhimurium and comingled with a pen of 27 broilers for 1 wk. Broilers were given 2 different organic acids, formic and propionic acid at different concentrations (either 4 kg/ton or 6 kg/ton formic acid and 5 kg/ton or 10 kg/ton of propionic acid) for different lengths of time. Propionic acid was added to the diet for the entire 6 wk of grow-out or the final week of grow-out. Formic acid given only during the final week of grow-

out. By the end of grow-out, the broilers given propionic acid at 5 kg/ton for the entire grow-out period had no *Salmonella* recovery in the litter (pen) or in the ceca compared 5/30 positive ceca and 1/3 positive pens. In a similar experiment done before, formic acid was administered either in the feed only, water and the feed, water only or no acid (control) all equaling the same concentration (6 kg/ton), at 3 wk there was no difference in the colonization of *Salmonella* in the treatment groups, however by 6 wk, there was a lower recovery of *Salmonella* in the feed and water treatment group.

Adding specialized prebiotics may not be possible depending on the cost of the original sources of the compounds used, so research has also focused on examining dietary regimens. This has been the case for layer management practices in the poultry industry. In particular, molting diets for layer hens have been a focal point for development of these types of diets. Natural molt of hens is associated with the temporary interruption of egg production (Kuenzel 1993). Historically, the shortening of the natural molt and rejuvenation of hen flocks in poultry industry were achieved by withholding feed (Bell 2003). Feed deprivation was a procedure employed to achieve a rapid and economical new egg-laying cycle (Brake 1993, Holt et al. 1995) and could last anywhere from 4 to 14 days (Bell 2003, Webster 2003). Although possessing several management advantages, withholding feed to layers has become less popular due to a variety of animal and food safety issues (Holt 2003, Ricke 2003, Park et al. 2004)]. The avian microbial ecology may be altered during dietary stresses such as feed removal which in turn can lead to higher vulnerability of the host to pathogen infection and colonization (Holt 2003, Durant et al. 1999, Dunkley et al. 2007). Hens also become more susceptible to pathogen infection including *Salmonella* spp. since molted hens shed significantly more *S*. Enteritidis in their feces (Holt 1992, Holt 1993, Holt 1995). These findings suggested that complete removal

of feed promotes pathogen invasion in molted hens. Higher levels of *S*. Enteritidis can be translocated to the internal organs including liver, spleen, and ovaries (Thiagarajan et al 1994, Holt 1995 and Durant et al 1999). The mechanism of *Salmonella* moving throughout the body of a chicken is not fully understood. Generally though, The infection normally starts from the intestines, from there *Salmonella* can enter the blood circulation and spread to various organs such as the liver and spleen, leading to systemic infection.

Dietary fiber as a prebiotic may be utilized preferentially by *Lactobacillus* and *Bifidobacteria* species which leads to the production of lactic acid and SCFA, resulting in the maintenance of normal microbial populations, low pH and the prevention of the establishment of *Salmonella* in the GI tract (Kaplan et al. 2000, Fernandez et al. 2002). Dietary fiber also reduces passage rate. Diets that reduce the passage rate could be advantageous since this mechanism may prolong fermentation which in turn increases metabolites needed to maintain GI tract integrity. The altering of passage rate represents changing the amount of digesta that passes a point along the GI tract in a given time (Brant et al. 1958). Passage rate may vary in different segments of the GI tract and is dependent on the feed composition and texture (Dänicke et al. 1997, Mikkelsen et al. 2004). Adequate feed retention time is essential especially in the ceca in order to encourage microbial degradation for longer periods of time (McNab 1973) leading to the production of important metabolites, which subsequently maintain the integrity and an optimal range of microbial diversity.

Several high-fiber dietary approaches have been tested and can utilized as alternative molting diets to expedite an additional laying cycle for hens. This includes insoluble plant fiber such as cotton meal, which layers did not eat (Davis et al. 2002), wheat middling (Seo et al. 2001), and alfalfa (Woodward et al. 2003, Dunkley et al. 2007, McReynolds et al. 2005,

McReynolds et al. 2006). In addition, microorganisms that are indigenous to the GI tract of poultry have the potential to hydrolyze dietary fiber into oligosaccharides and other low molecular weight carbohydrates which leads to production of SCFA (Allen et al. 1997, , Dunkley et al. 2007). Alfalfa as an example may have advantages associated with the fermentation properties by cecal microflora that are capable of limiting *in vitro* growth of *S*. Typhimurium and has been shown to limit *in vivo S*. Enteritidis colonization in laying hens (Woodward et al 2003, Donalson et al. 2008). An *in-vitro* study examined the fermentation of alfalfa and layer feed incubated with chicken cecal content in rumen fluid using nitro compounds and indicated that both feed materials influenced SCFA production with acetate being the predominant component (Saengkerdsub et al. 2006). High-fiber feed substrates have also been observed to influence microbial diversity and stimulate SCFA production when incubated with chicken cecal inocula *in vitro* (Dunkley et al. 2007).

In order to derive maximum benefit from fermentable high-fiber prebiotic sources, physical modification may also be necessary to derive uniform particle size. Coarsely ground mash over whole grain wheat has been demonstrated to be effective on the physiological function on GI tract of broiler birds. The increase in feed structure (whole grain) caused an increase in gizzard size (Svihus et al. 1997, Nir et al. 1994a, Nir et al. 1994b, Enberg et al. 1992) while a reduction in gizzard pH and an increase in small intestinal pH were observed with an increase of the grain particle size (Nir et al. 1994b, Enberg et al. 1992).

Numerous studies have been carried out to evaluate the effects of feed structure on performance of poultry. Previous research also has shown with the inclusion of whole grains to feed instead of pelleted compound feed was shown to increase feed conversion and growth of broilers. Furthermore, whole wheat feeding significantly increased gizzard weight, increased

retention time, and reduced pH in gizzard contents compared to pellet fed birds (Engberg et al. 2004 and Bjerrum et al. 2005) which in turn decreased the *Salmonella* population. In addition, uniform particle size was shown to contribute to the development and integrity of the GI tract which subsequently enhanced gut motility and backflow mechanisms in poultry including reverse peristalsis from the cloaca to the ceca (Karasawa 1989). Alfalfa when fed in a crumble form appears to support microflora that are accompanied by increased production of SCFA in a pattern similar to a grain-based diet (Dunkley et al. 2007). While feed removal resulted in decreased fermentation capacity (Woodward et al. 2003), the negative effect was neutralized by hens fed alfalfa crumbles as acetate, propionate and butyrate were observed to be the most pronounced SCFA in feces and ceca (Dunkley et al 2007).

Charcoal as a prebiotic

Charcoal is produced by heating organic products (wood, bamboo, coconut shells etc) in airtight ovens or retorts, in chambers with various gases, or in kilns (Belinger 1952) supplied with limited and controlled amounts of air. The carbonization or "coaling," temperatures used control the amounts of volatiles or smoky materials remaining in the charcoal (Hawley 1923). If coaling temperatures are too low, excessive amounts of volatiles will remain in the charcoal and cause heavy smoke when it burns. The amount of coal that is made from a given amount of organic material is dependent upon the amount of carbon in the wood and the carbonization conditions. On average, carbon constitutes about 50% of the dry weight of the wood. Good-quality charcoal burns cleanly and has a heat value of about 13,000 British thermal units (13,800 kilojoules) per pound (Belinger 1952). The organic matter is dried then high-temperature heating by all methods breaks down organic matter into gases, a watery tar mixture, and the familiar solid carbon material commonly known as charcoal. The yield of charcoal varies with major

changes of wood moisture content. Generally the moisture of wood makes a difference on the conversion to charcoal. For example, wood at 20 to 35% moisture content requires the shortest coaling cycle; wood at 35 to 45% requires a medium coaling cycle while wood at a moisture content of 45% or more requires the longest coaling cycle. Wood with less than 20% of moisture is likely to coal too rapidly, and care must be taken to avoid development of excessively high temperatures. Also a general rule, then, the denser or heavier the wood used, the greater will be the weight yield (USDA-Forest Services, 1961)

Charcoal is porous which creates a large internal surface area. Charcoal's surface area may vary from 500 $m^2 g^{-1}$ (with lignin as starting material) to 2000 $m^2 g^{-1}$ (oil as starting material) (Diamadopoulus et al. 1992). Charcoal can reach to an internal surface area of 3000 $m^2 g^{-1}$(Rodrígues-Reinoso, 1997; Bansal et al., 1988) that can be materially modified by activation to adsorb greater amounts of these materials. Charcoal becomes "activated" when heated at high temperature in the presence of air, steam, carbon dioxide, or combinations of these gases. Activated charcoal, also commonly known as activated carbon can be prepared from a wide variety of raw materials (Dąbrowski, 2001). The ingredients are usually have a high carbon content and a low inorganic content. The materials to make activated charcoal should be easily activated and should have low degradation by aging (Moreno-Castilla and Rivera-Utrilla, 2001). Coal is the most commonly used precursor, mainly due to its low cost and large supply (Ahmadpour and Do, 1996).

Charcoal when its ground creates a very fine, odorless, tasteless black powder which has a high adsorbance level for many toxins gases, drugs, fat and fat-soluble substrates without any specific action (Osol, 1975). Adsorption is the process by which liquid, gaseous molecules or

bacteria in this case are concentrated on a solid surface, in this case activated carbon (activated charcoal). This is different from absorption, where molecules are taken up by a liquid or gas.

Activated carbon is utilized for filtration throughout the world. In fact, around 80% of the world production of activated charcoal is used in liquid-phase applications (Moreno-Castilla and Rivera-Utrilla, 2001). Also, the treatment of wastewater and contaminated groundwater using activated charcoal is increasing throughout the world as a result of the limited sources of water supply (Meidl, 1997). In such treatments, activated charcoal is normally used as a primary treatment, preceding other purification processes, or as a final tertiary or advanced treatment of liquid, gaseous and heavy trace metals (Lalhruaitluanga et al. 2010). Adsorption of Pb(II) at different levels of Melocanna baccifera (bamboo) charcoal (0.1 to 0.5 g/100 mL) was analyzed at a constant concentration of metallic lead in solution. The percentage of adsorption of Pb(II) increased from 15 to 82% as the levels of charcoal increased. Similarly, the percentage of metallic lead adsorption was increased from 59 to 99% for Melocanna baccifera activated charcoal with increased level of charcoal (Lalhraitluaga et al., 2010). Adsorption of metallic ions from aqueous solution is far from being a straightforward process. Generally though, metallic ions have small size, being frequently charged in solution; therefore, the predominant interactions in their adsorption process on activated carbon are of electrostatic nature (López-Ramón et al., 2002). Wood makes a particularly porous activated carbon (Wu and Tseng, 2006).

Adsorption of chemicals onto charcoal depends on the pore size, surface area, the dose of charcoal (Diamadopoulus et al. 1992). The adsorption of aflatoxins and mycotoxins has been more well studied using activated charcoal. Hatch et al. (1984) showed that giving activated charcoal with a lethal dose of aflatoxin B_1 to goats resulted in an average percentage of liver destruction of only 3% compared to 25% in the non-charcoal treated goats. They showed the

therapeutic effect of activated charcoal in a high dose challenge of aflatoxin B_1 was due to the adsorptive properties of the charcoal. This hypothesis was further supported by the fact that the feces from charcoal treated animals' contained larger concentrations of aflatoxin B_1 while the non-treated group had only slight amounts of aflatoxin in their feces. The capacity of granulated activated carbon to adsorb monohidroxilated phenols catechol, resorcinol and hydroquinone depends on the pH of the solution; the quantities adsorbed in accordance with the adsorption isotherms decrease when the pH is increased from 7 to 11 and the maximum adsorption is obtained at pH 7.

In poultry, the use of charcoal has limited available published material. Kutlu et al. 2001 explained in their research that some of these ingredients are not cited in scientific literature but can be used locally. Specifically, local poultry producers claim that including 20-50 g/kg diet of oak charcoal prevents fatness and improves overall performance (Kutlu et al. 2001). Their work showed that adding 50g/kg of wood activated charcoal to broiler starter diets (8-28 d) resulted in a lower feed conversion ratio (1.43) compared to no additional charcoal (1.50), in their first experiment. The broilers however were not significantly different in feed intake or feed conversion by the end of the grow-out (49 d). Carcass weights were significantly higher (1845 g versus 1716 g in basal control) with broilers fed 25g/kg during the starter phase and no charcoal during the finisher phase. The inclusion increased the carcass ash content, which may have been an indicator of increased mineral retention as mineral intake increased. They theorized that the charcoal improved the condition of digestion at an early age. When feeding charcoal and its effect on growth of broilers eating feed with aflatoxins, there are mixed results. Edrington et al. 1997 fed 0.5% superactivated charcoal (decreased particle size of activated charcoal to increase surface area and chemically modified (Requa, Inc., Greenwich, CT) the charcoal) to male

broilers for 21 days, there was no significant effect on body weight gain, feed to gain ratio, and lesions caused by the aflatoxin compared to those given the aflatoxin feed alone. However, Dalvi et al. 1984 and Jindal et al 1994 reported improved body weight gains and feed intake with broilers fed an addition of activated charcoal when the feed contained aflatoxins compared to the group consuming aflatoxins alone. However, broilers weighed at a maximum average of 832 g (basal control group) without measuring feed conversion (Jindal et al. 1994).

The adsorption of bacteria using charcoal has limited available published research. A number of publications reference papers from Hoshi et al. 1991 (Chu et al 2013, Kutlu et al 2001, Ayanwale et al 2006) and Nikoleava 1994 that may assume to answer questions concerning the change of microflora and gut environment of food animals when given charcoal. These publications are not accessible in English (Japanese or Russsian) and Hoshi et al. 1994 had an animal sample size of 4. However recent research has looked at interactions of antibiotics and the influence on bacterial growth. Oral gavage of charcoal accompanying cefotaxime (CTX)-resistant *Klebsiella pneumoniae* strain of bacteria has been reported to reduce the fecal recovery of CTX-resistant *Klebsiella pneumoniae* bacteria by 3 $\log_{10}$ by the adsorption of the antibiotic CTX within the colon of mice (Grall et al., 2013). The use of charcoal to adsorb food-borne bacteria within the intestines of poultry has limited available published research. However, the adsorbance of *Salmonella* by charcoal in-vitro has been reported. Wateri et. al. (2005) showed that *Salmonella* Enteritidis was adsorbed significantly more effectively with the addition of charcoal compared to a common intestinal bacteria, *Enterococcus faecium*. Their theory is that *Salmonella* being the ideal size, 2 by 0.5 µm, may be one of the causations for activated charcoal to readily adsorb *Salmonella*. Charcoal alone has not been tested or reported for adsorbance of *Salmonella* in a large scale in-vivo in poultry during production.

Sources of *Salmonella*: Litter

Poultry litter when recycled has been identified as a possible source of *Salmonella* on the carcass of broilers (Reiber et al 1990). Sampling the poultry-house litter has been used to indicate Salmonella of flocks for decades (Kingston, 1981). The technique that is the most widely accepted are direct culture of litter and drag swabbing the floor of the poultry house (Bhatia et al., 1980; Kingston, 1981; Mallinson et al. 1989; Opara et al., 1992; Caldwell et al., 1994). Using the intentionally stepped on drag swab method, the drag swab is able to recover up to 64% more positive samples otherwise would have been negative under conventional method (Buhr, 2007). The increased surface area and the direct contact with fecal material is increased when the drag swab is being intentionally stepped on.

Depending on litter type, broiler chicks have the potential to consume up to 6% of wood fiber during the first 7 d of life post placement(Malone et al. 1983). As the seeders defecate on the litter and in the feed trays, penmates are able to consume fecal material. Broiler chickens can harbor *Salmonella* spp. from hatch through grow-out although numbers of birds shedding *Salmonella* appear to decline over time. Additionally, *Salmonella* in poultry appears to have a commensal relationship as the bird's health is not impaired and birds do little to exclude the organism once *Salmonella* is established. Direct oral challenge may result in higher numbers of *Salmonella* being introduced into each bird versus using seeders to spread *Salmonella*. Therefore, colonization levels may increase as numbers of *Salmonella* within the host increases.

A potential parameter in the growth of Salmonella of poultry litter surface is water activity (Opara et al 1992), which represents the ratio of water vapor pressure of the litter to the water pressure of pure water. The water activity scale extends from 0 to 1.0, dry to pure water. In turkey litter, *Salmonella* populations exhibited growth (approximately 2 log) with little decline

up to 42 d in litter environments of pH 7 and 9 and a water activity of 0.96. As water activity and pH levels decreased, *Salmonella* populations declined at a pH 4 and water activity of 0.84 (Payne et al. 2007). Generally, as the flock ages, *Salmonella* levels decrease due to low water activity and high pH from ammonia in the litter (Turnbull and Snoeyenbos 1973).

Salmonella present in the litter maybe difficult to recover depending on the sampling technique . In Buhr et al. 2007 study for example, direct litter sampling was not sufficient to predict external carcass contamination. Chicks arriving from the hatchery have high levels of presumptive coli-aerogenes bacteria such as *Streptococcus faecales* and *Streptococcus faceium.* However no *lactobacilli* was found (Barnes. et al 1980). Though 3 days later, *Lactobacillus* ssp had reached levels of 9 $\log_{10}$/g. This many indicate that poultry rapidly develop a resistance of *Salmonella* colonizing.

According to Cray et. al 2001, when *Salmonella* is shedding from broiler feces, challenged 10^6 colony forming unit (CFU) *Salmonella* Typhimurium DT104 (a specific subspecies within serovar *Salmonella* Typhimurium and is a specific strain within DT104 whose full name is *Salmonella enterica* serovar Typhimurium variant DT104 R-ACSSuT) broilers compared to their penmates, were not different in colonization of mean $\log_{10}$ CFU/g at 6, 9, 13, 17 and 20 days post challenge. As chicks age, once exposed to *Salmonella*, both seeders and penmates begin to shed it out of the body into the litter. Depending on how well the strain of *Salmonella* colonizes, environmental conditions and length of time chicks are exposed to a challenged seeder, broilers may have some *Salmonella* back in the background. Cray et al 2001 data indicated that following exposure of day-of-hatch chicks to *Salmonella* Typhimurium DT104, either by oral challenge or indirect contact (commingling with a oral challenged chick),

chicks will remain colonized with DT104 throughout the grow-out period, the colonization percentage has a decreasing trend over time (by 3 weeks).

Poultry litter has several species of bacteria. Lu et al 2003 detected many bacterial sequences for organisms in used poultry, such as *Globicatella sulfidofaciens, Corynebacterium ammoniagenes*, *Corynebacterium urealyticum*, *Clostridium aminovalericum*, *Arthrobacter* sp., and *Denitrobacter permanens,* that may be involved in the degradation of wood shavings in the litter and cycling of nitrogen and sulfur. However, using specific PCR targeting, they did not detect the presence of *Salmonella*, pathogenic *Escherichia coli*, *Campylobacter* spp., *Yersinia* spp., *Listeria* spp., or toxigenic staphylococci. Gram-negative bacteria, *Salmonella spp*, *Escherichia coli* and *Campylobacter,* that are often human pathogenic, account for approximately 0.1% of the microflora of the ceca and ileum of poultry (Lu et al 2003).

Theories of microbial growth inhibition by organic acids was explained by the ability of these acids to pass across the cell membrane, dissociate in the more alkaline interior and acidify the cell cytoplasm (Kashket, 1987). However some bacteria are more sensitive than others. It has been assumed that bacteria maintained a slightly alkaline intracellular pH, but this assumption was largely based on work with laboratory cultures of *E. coli* (Padan et al., 1981). Many fermentative bacteria have the ability to let their intracellular pH decline when the extracellular pH becomes highly acidic. The effects of organic acids on the epithelial invasion of *Salmonella* may be explained by changes in *Salmonella* pathogenicity island 1 (SPI-1) expression. SPI-1 is a secretion system under Type 3 secretion system (TTSS) identified in *Salmonella* enterica species serovar Typhimurium. This allows the bacteria to secrete and inject bacterial toxins (effector proteins) directly into the cytosol of the host cells, where the toxins

induce responses (Ehrbar et al. 2003). The responses include inflammatory responses and invasion of the intestinal epithelial cells (Galan 2001, Santos et al. 2001, Wallis et al 2000).

Salmonella is an opportunistic intracellular pathogen that has an elaborate set of virulence genes. These genes enable the bacterium to adapt to the environment and move between various micro-niches within a host. An early step in the pathogenesis of *Salmonella* is the penetration of intestinal epithelium (Lostroh et al. 2001) promoted by SPI-1 invasion genes. Generally, SPI-1 has genes encoding regulatory proteins, structural components of a needle complex and additional effector proteins. Bacterial effector proteins facilitate the entry of *Salmonella* into the cytosol of epithelial cells, by inducing actin rearrangements that lead to uptake of the bacteria. When *S.* Typhimurium was pre-incubated in growth media supplemented with various concentrations of butyrate and propionate, epithelial cell invasion was suppressed. However, if the cells were preincubated in media supplemented with acetate, invasion was still observed (Durant et al. 1999; Lawhon et al. 2002; Van Immerseel et al. 2004). Similar results were obtained with *S.* Enteritidis when primary cecal epithelial cells of the chicken were employed (Van Immerseel et al. 2004).

In a large-scale study (Humphrey & Lanning, 1988) the number of *Salmonella*-positive breeder feed samples decreased from 4.1 to 1.1% after the feed was supplemented with 0.5% formic acid. The antibacterial activities of organic acids were dependent on temperature and moisture. Since the water content of poultry feed is generally low, the action of the acids is not always optimal, and it is not clear whether in-feed effects are the major reason for protection (Hinton 1990). Acid concentrations increase in the crop, and this antibacterial action could aid in controlling infection caused by horizontal transmission. Therefore, when acid-treated feed is given to poultry, the activity of the SCFA should increase. It appears that supplemental acids are

most adapted to affect in the crop and gizzard rather than in the intestine. This point is illustrated in a study of Thompson and Hinton , who fed laying hens a feed supplemented with a commercial mixture of formic and propionic acids. In these animals, pH values of the crop, gizzard, jejunum, cecum and colon were not altered relative to control animals, but formic acid and propionic acid concentrations in the crop and gizzard were significantly increased (Thompson et al. 1997). The lactic acid concentration in the crop decreased significantly, suggesting that lactobacilli were either inhibited or killed (Thompson et al. 1997). These results followed a study by Hume and coauthors where large increases in propionic acid concentration in the crop of 4 d broilers were detected, when propionic acid was added to poultry feed, despite no observed changes in crop pH. Cecal SCFA patterns were not affected (Hume et al., 1993).

Many studies examined the effects of supplemental acids on *Salmonella* colonization of poultry tissues. Actions of formic and propionic acids were variable. In a small-scale field trial, formic acid controlled shedding and cecal colonization by *Salmonella* in naturally infected animals. Indeed, 50% of all control animals had *Salmonella*-positive cloacal swabs and cecal content samples, but *Salmonella* could not be detected in animals that consumed significant concentrations of formic acid (Hinton et al. 1985). In a 3 year study, the cumulative number of infections of newly hatched chicks with *Salmonella* decreased after breeder stocks were given formic-acid-treated feed (Humphrey et al. 1988). Breeders that received acidified feed had fewer numbers of *Salmonella* in the breeder litter (4.3 versus 1.4%), hatchery waste (15.3 versus 1.2%) and insert chick box paper samples (4.6 versus 1.4%). These decreases were evident from the moment the breeders received acidified feed and illustrate the effects on vertical transmission (Humphrey et al. 1988). The most striking proof of the efficacy of formic and propionic acids as feed additives to control *Salmonella* (Hinton et al. 1988). In three independent experiments, no

artificial infections or feed inoculations with *Salmonella* were performed. Formic acid-supplemented feed, given from the day of hatch, decreased the number of positive feces and cecal content samples dramatically. The control groups had 25, 27 and 60% *Salmonella*-positive fecal samples, but the treatment groups had 3, 0 and 0% (Hinton et al. 1988). When the formic-acid-treated feed was given at a later age (16 or 32 d), no differences were detected between control and treated groups. This illustrates that preventing initial colonization of *Salmonella* is most important. Once an infection is established, it is very difficult to counteract using acid-treated feed, at least in the same production round.

Sources of *Salmonella*: Crop

The crop's primary function for poultry is feed storage prior going to the gizzard and proventriculous. However, some carbohydrate digestion may occur in the crop due to the presence of amylase activity (Phillips and Fuller 1983). Amylase activity at this site comes from salivary secretions, intestinal reflux or plant and/or bacterial sources. Bolton reported that starch is hydrolyzed within the crop where it can either be absorbed; converted to either alcohol, lactic acid or other acids; or transported down the gastrointestinal tract (Bolton 1965) and substantial amylolysis in the crop (Pinchasov and Noy 1994). While absorption of sugars from the crop appears possible, it is probably minimal. The crop is not essential for normal growth when access to food is sufficient. Performing cropectomies has no effect on growth rate of ad libitum fed chicks, but it does decrease growth rate when food intake is limited. This supports the view that primary function of the crop is food storage, and it is not essential for digestion (Chaplin et al. 1992, Richardson 1970).

Lactic acid bacteria is among the major bacteria responsible for fermenting feed in the

crop of poultry (Fuller 1977). Intestinal bacteria isolated from poultry ferment feed to produce concentrations of lactic acid that reduce the pH to levels that can inhibit the growth of *S.* Typhimurium and Escherichia coli O157:H7 in vitro (Hinton et al 1992). Hargis and coauthors reported that broiler crops were frequently contaminated with *Salmonella* at commercial processing. In that study, crops were observed to be 3.5 times more likely to be contaminated with *Salmonella* than ceca, and crops ruptured 85 times more frequently than ceca during processing (Hargis et al. 1995). Additionally, the incidence of *Salmonella* recovery from crops has been reported to significantly increase following feed withdrawal in both experimental and commercial settings (Humphrey et al. 1993, Ramirez et al. 1997).

The purpose of feed withdrawal is to empty the alimentary tract to reduce contamination of carcasses when processed. Increasing pH during feed withdrawal has been attributed to decreased *Lactobacillus* fermentation within the crop and is associated with increased *Salmonella* recovery following feed withdrawal (Humphrey et al., 1993). Emptying the crop, the absence of feed will increase the pH because the lactic acid that ferments feed will not be there, where they lower the pH of the environment. *Salmonella* generally does not thrive in a low pH environment. For example acidic fruit such as grapefruit (pH 2.9-3.2) in a study reduced levels of *S*. Typhimurium adherence to cultured intestinal epithelial cells by 66% compared to the control by incubation for 24 h (Yin et al. 2012). Farner (1943) found the pH of certain portions of the chicken's digestive tract varied widely (n=20): crop 4.5, proventriculous 4.4; gizzard 2.6; small intestine 5.8-6.4 and large intestine 6.3. The pH data was obtained from 2-year-old females and 1-year old males. Farner explained that since there was no significant difference between sexes that he combined the data. The data is limited on the pH of crops of broilers versus layers. Reports found in the literature (Johnson and Griffith 1970) have indicated that the

generally accepted time required for food passage in the laying hen is approximately 4 hours. However this may be different for broilers.

Sources of *Salmonella*: Other portions of the Alimentary Tract

Barrow and coauthors found that if chicks are inoculated at d 1 10^5 cfu *S.* Typhimurium *S.* menston, *S.* chloerae-suits at day 1, all *Salmonella* serovars can be found in the ceca and cloaca between $\log_{10}$ 8.2 to 6.3, 3.9 5.8 from 1 to 34 d post oral challenge (Barrow et al. 1988). While chickens (Light Sussex) at 21 d given 10^8 cfu of *Salmonella* Typhimurium, had a challenge recovering cecal and cloacal after 7 d, after 14 d no recovery in the cloaca and after 21 d no recovery in the ceca (no recovery in crop, gizzard, duodenum, jejunum and ileum) (Barrow et al. 1988)

The avian small intestines consist of the duodenal loop jejunum and ileum. The small intestine is the principal site of chemical digestion, involving enzymes of both intestinal and pancreatic origin. It also secretes hormones that are primarily involved in regulation of gastric and intestinal actions. Additionally, most nutrient absorption occurs in the small intestine (Herpol 1966, Herpol 1967). Farner 1942 tested the contents of feed to find the hydrogen ion concentration of 1 year old male and 2 year old female chickens and the crop ranged from 4.74-4.54 (n=20), duodenum 5.68-6.07, ceca 5.6-5.83, gizzard 2.46-2.79, proventriculous 4.33-4.51 colon 6.08-6.58. *Salmonella* is not suited for an acidic environment. The infection is initiated by the adherence of *S.* Typhimurium to the intestinal epithelium followed by invasion and destruction of M cells (specialized epithelial cells associated with the intestinal barrier function) and enterocytes, resulting in disruption of the integrity of the mucosal surface and entry to the underlying tissue (Jones et al., 1994). Colonization of the intestine by *S.* Typhimurium is an

essential early stage of pathogenesis (Wagner and Hensel, 2011). Adherence of *S.* Typhimurium to cultured intestinal epithelial cells was reduced to levels of 3.3–61% of the control by incubation in grapefruit juices (pH 2.9–3.2), apple cider (pH 3.5) and TSB at pH 3.5 for 24 h. These data suggest that the inhibitory effect of grapefruit juice and apple cider on the survival and adherence of *S.* Typhimurium may be largely due to the low pH in these juices during 2 h and 5 h incubation (Yin et al. 2012).

Sources of *Salmonella*: Breast skin

Bacterial contamination of the external surface of processed poultry carcasses can originate from digesta or feces excreted from the alimentary tract during grow-out, transportation, or processing (Oosterom et al., 1983; Genigeorgis et al., 1986; Izat et al., 1988; Hargis et al., 1995; Stern et al., 1995; Byrd et al., 1998; Berrang et al., 2002). There is some controversy of feather follicles being a potential reservoir of bacteria because "bacteria contained in dirty water (from the scalder) may be massaged into the skin and open feather follicles" (Russell 2012). Barnes et al. (1973) pointed out that maceration of poultry skin samples yields higher numbers of bacteria than other sampling techniques. These authors speculate that the higher recovery was “probably due to the bacteria growing down in the feather follicles rather than at the surface of the skin.” Broiler carcasses are typically scalded for 2 to 3 min in water reported to contain about 13 CFU of *Salmonella* per 100 mL, on the occasion when *Salmonella* is present (Humphrey and Lanning, 1987; Cason et al., 2000).

Genetically feathered and featherless sibling broilers (Buhr et al. 2003) selected for matched body weight (BW**)** were killed, scalded, and immediately after defeathering, breast skin was aseptically removed to see if feathers and empty feather follicles increased the prevalence of

bacteria from a carcass. Bacteria *Campylobacter*, *Escherichia coli*, total coliforms and total aerobic bacteria were enumerated. In their first trial, the levels of all bacteria recovered did not differ between feathered and featherless carcasses. In their second trial, the carcasses that had vents plugged and sutured had lower levels of all four types of bacteria (differences of *Campylobacter* $\log_{10}$ 0.7 cfu/mL, coliform $\log_{10}$ 1.8, E. coli $\log_{10}$ 1.7, and total aerobic bacteria $\log_{10}$ 0.5) than those carcasses with open vents showing that cloacal contents expelling out onto carcasses during defeathering is more of a potential contamination than the presence of feather follicles. The lower levels of bacteria recovered from carcasses with the vents plugged and sutured during picking enabled detection of small but significant differences between feathered and featherless carcasses. The level of coliform and *E. coli* recovered was slightly higher by $\log_{10}$ 0.7 cfu for feathered carcasses, but featherless carcasses had marginally higher levels of total aerobic bacteria by $\log_{10}$ 0.4 cfu. Feathered and featherless carcasses with open vents during picking did not differ in the levels of recovery of coliform, *E. coli*, and total aerobic bacteria from breast skin. Overall, this study indicates that bacteria, including *Salmonella* is not being massaged into the feather follicles of broilers.

Fecal shedding from positive *Salmonella* flocks are in contact throughout transport facilitates. Feces from *Salmonella* positive flocks cross-contamination between *Salmonella*-free carcasses and equipment during processing (McCrea et al., 2006). Feed withdrawal, loading, and transportation from farm to slaughter-house are known to be stressful for poultry (Mulder 1995, Burkholder et al. 2008; Scherer et al 2008). Specifically the transport to the slaughter-house increases the prevalence of *Salmonella* positive poultry due to fecal contamination of skin and feathers by neighboring infected birds during shipping (Sadler et al. 2002, Heyndrickx et al 2002, Marin et al. 2009). These authors suggest that the lowering of farm prevalence of

Salmonella during transport are important strategies to lower the risk of contaminated meat products entering the food chain.

Although broiler carcasses with feces-soiled feathers and skin had higher levels of coliform and *Escherichia coli* than carcasses with clean feathers prior to scalding and picking, after defeathering all carcasses had lower levels of these bacteria, and the levels no longer differed between the dirty and clean carcasses (Kotula and Pandya, 1995; Buhr et al., 2000). Therefore, broilers may enter the processing plant with gross contamination with feces and bacteria on skin, feet, and feather surfaces, but the level of bacteria typically decreases substantially as the carcasses progress through processing stages (Oosterom et al., 1983; Lillard, 1989, 1990; Berrang and Dickens, 2000).

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CHAPTER 3

EVALUATING THE ADDTION OF CHARCOALS TO BROILER DIETS ON THE RECOVERY OF SALMONELLA TYPHIMURIUM DURING GROW-OUT AND PROCESSING[1]

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Abstract

Two experiments evaluated charcoals added to the feed on the recovery of *Salmonella* in broilers during grow-out and processing. In experiment 1, 2two "seeder" chicks were inoculated with *Salmonella* Typhimurium and placed with 19 penmates/pen in 32 pens. Treatments were: basal control, 0.3% bamboo charcoal, 0.6% bamboo charcoal or 0.12% Aromabiotic® (8 pens/TRT). The ceca from seeders and penmates were sampled to confirm *Salmonella* colonization at 3, 4 and 6 wk, and pen litter was sampled weekly. At 3 wk, charcoal fed chicks had significantly lower recovery of *Salmonella* via direct plating but no difference at 4 wk. At 6 wk, broilers fed Aromabiotic had significantly lower recovery of *Salmonella* with enrichment. In experiment 2 the treatments were: basal control; 0.3% bamboo charcoal; 0.3% activated bamboo charcoal or 0.3% pine charcoal (10 pens/TRT). At placement, 2 seeders were challenged with *Salmonella* and commingled with penmates. Seeder and penmate ceca were sampled at 1 and 2 wk and ceca from 5 penmates/pen at 3 to 6 wk. The pH of the crop and duodenum was measured weekly from 1 penmate/pen, and litter was sampled weekly. At the end of grow-out, broilers were processed on two consecutive days. Results showed that penmates were colonized at 1 and 2 wk. Cecal *Salmonella* showed no difference except at 4 wk, when activated bamboo charcoal had a 14% lower recovery of *Salmonella* with enrichment compared to the control. Similar to experiment 1, the recovery of *Salmonella* from the litter was not significantly different between treatments, however an overall decrease in recovery by 4 wk with direct plating was noted. The pH of the duodenum and the crop were not different among treatments. Crop pH from all treatments were significantly higher at wk 1 compared to wk 2 thru 6. At wk 3, 4, and 5 *Salmonella* was dislodged from the broiler's ceca. Although litter recovery of *Salmonella* was

not significant among treatments, charcoal had minimal effect on the recovery in the ceca, a significant reduction on the recovery from breast skin (20% decrease) was observed.
Key Words: *Salmonella*, Broilers, Charcoal, Ceca

Introduction

The contamination of broiler carcasses with *Salmonella* remains a tremendous problem and concern for the poultry industry and regulatory agencies. The process of growing broiler chickens gives rise for opportunities of pathogens such as *Salmonella* to be transferred from the environment to the chick and then from bird to bird. Cox and coauthors suggested that the newly hatched chick may be exposed to significant levels of *Salmonella* from an assortment of sources such as the hatching cabinet, hatchery environment, and the broiler house (Cox et al. 1996). Once *Salmonella* reaches the primary site of colonization, the ceca of a young chick (Milner and Shaffer 1952, Brownell et al 1969, Barrow et al., 1988, Fanelli et al 1971, Snoeyenbos et al 1982), and it is speculated that *Salmonella* may attach to the intestinal mucosa to colonize (Barrow et al. 1988). By the time broilers reach the market age and weight, *Salmonella* may be recovered at lower numbers from the crop or ceca but can remain on the carcass and be recovered after processing on the final product. Generally, the highest levels of intestinal colonization of Salmonella occurs between wk 2 and 3 of grow-out, after which there is typically a gradual decline in frequency until the time of processing (Bailey 1993). A study sampling 56 broiler flocks and collected in 6 processing plants and although only 7 (13%) broiler flocks were determined to be colonized with *Salmonella* at slaughter in the ceca contents, carcasses from 31 (55%) of the broiler flocks were contaminated with *Salmonella* after slaughter (Rasschaert et al. 2008). If the prevalence of *Salmonella* could be lowered in the broilers by the

time they arrive at the processing plant, then the risk of contaminating the carcass may be reduced significantly.

Prebiotics added to feed are considered to be a possible answer to contribute to lowering the recovery of *Salmonella* without the use of antibiotics, which are not allowed in the European Union (European Commission, 2011). Prebiotics may work by either binding to the bacterial pathogen in the intestinal lumen and blocking the adhesion of bacteria to the epithelia cell surface (Spring et al., 2000), or prebiotics may provide a substrate for the metabolism and growth of intestinal flora, thus inhibiting pathogen colonization by competitive exclusion (Suskovic et al., 2001). Charcoal is a carbanecaous material that has high porosity and immense internal surface area. Charcoal generally refers to the carbonaceous residue of wood; cellulose, coconut shells or other various industrial wastes left after heating organic matter in absence of oxygen. Charcoal can be ground to a very fine powder that is odorless and tasteless, and acts as an absorbent for many toxins, including aflatoxins, other mycotoxins, dissolves gases, drugs, fat and fat-soluble substances (Dalvi et al 1984a, Dalvi & McGowan 1984b, Jindal et al., 1994; Jindal and MahiPal, 1999, Sands et al 1976.). Adsorbance by activated charcoal depends on pore size and surface area, concentration, and chemical nature of the source of the charcoal (Diamadopoulos et. al., 1992). Hatch et al. (1984) showed that giving activated charcoal with a lethal dose of aflatoxin B_1 to goats resulted in an average percentage of liver destruction of only 3% compared to 25% in the non-charcoal treated goats. They attributed the therapeutic effect of activated charcoal in a high dose challenge of aflatoxin B_1 was due to the adsorptive properties of the charcoal. This hypothesis was further supported by the fact that the feces from charcoal treated animals' contained larger concentrations of aflatoxin B_1 while the non treated group had only slight amounts of aflatoxin in their feces.

Charcoal is considered to be among the best currently available substances for removing low solubility contaminants for water treatment, including trace metals (Lalhraitluaga et al., 2010). Adsorption of Pb (II) at different levels of Melocanna baccifera (bamboo) charcoal (0.1 to 0.5 g/100 mL) was analyzed at a constant concentration of metallic lead in solution. The percentage of adsorption of Pb (II) increased from 15 to 82% as the levels of charcoal increased. Similarly, the percentage of metallic lead adsorption was increased from 59 to 99% for Melocanna baccifera activated charcoal with increasing levels of charcoal. This can be explained as charcoal adsorbent level increased, more and more surface area was available which exposed more active sites for binding of metallic ions. Charcoal's adsorbance ability of bacteria has limited reliable research. Oral gavage of charcoal accompanying cefotaxime (CTX)-resistant *Klebsiella pneumoniae* strain bacteria has been reported to reduce the fecal recovery of CTX-resistant *Klebsiella pneumoniae* bacteria by 3 $\log_{10}$ in the colon of mice (Grall et al., 2013). The use of charcoal to adsorb food-borne bacteria within the intestines of poultry has limited available published research. However, the adsorbance of *Salmonella* by charcoal in-vitro has been reported. Wateri et. al. (2005) showed that *Salmonella* Enteritidis was adsorbed significantly more effectively with the addition of charcoal compared to a common intestinal bacteria, *Enterococcus faecium*. Their theory is that *Salmonella* being the ideal size, 2 by 0.5 µm, may be one of the causations for activated charcoal to readily adsorb *Salmonella*. Charcoal alone has not been tested or reported for adsorbance of *Salmonella* in a large scale in-vivo in poultry during production.

The objective of these studies was to first evaluate the colonization and recovery of *Salmonella* Typhimurium using different concentrations of bamboo charcoal added to broiler feeds. The second experiment explored the use of different types of charcoal, pine charcoal or

bamboo charcoal (activated and non-activated) on the colonization and recovery of *Salmonella*, their effects on pH of the crop and duodenum, and at the end of grow-out the impact on residual *Salmonella* on the breast skin of the carcasses post defeathering.

Materials and Methods: General Animal Care and Use and Biosecurity for the use of *Salmonella*

The nalidixic acid resistant marker strain of *Salmonella* Typhimurium was a natural strain of *Salmonella* that was selected for resistance to nalidixic acid enabling recovery and the exclusion of any potential environmental *Salmonella*. This marker strain was not genetically altered by any recombinant techniques and is non-pathogenic for poultry. The protocols followed for these experiments were approved by the University of Georgia Animal Care and Use and the Biosafety Committees.

The door to the experimental rooms had a standard biohazard caution sign placed on the outside. Access was key-pad restricted, only providing the principal investigators and other researchers access. Gloves (latex or nitrile), disposable plastic boot covers, face masks, eye-goggles, antibacterial gel, disinfect spray (70% ETOH) and a portable eyewash station were provided and remained near the exit of the experimental room. Before contact with the birds and entering individual pens, researchers donned boot covers and gloves. For experimental purposes the plastic boot covers were changed before stepping into each pen, and used boots were removed upon leaving the pen or the experimental room. Dead birds were removed from a pen and placed into individual plastic bags and the weight recorded. Dead birds, all left over feed, and used disposable items were put into a container labeled with a biohazard symbol and incinerated. Researchers were responsible for daily observation of the birds. They followed the

University of Georgia Poultry Research Center standard operating procedures and used daily record forms/notebooks (maintained within each animal room) for collecting data.

Materials and Methods Experiment 1

Salmonella Inoculum, Animal Care, and Housing. The selected strain of nalidixic acid resistant *Salmonella* Typhimurium developed in Dr. Nelson Cox's laboratory at the USDA-ARS Russell Research Center in Athens, GA was used for two experiments. *Salmonella* glycerol stock was streaked onto Brilliant Green Sulfa (BGS; Neogen, Lansing, MI) agar plates containing nalidixic acid (Sigma, St. Louis, MO) at 200 μg/mL, and the plates were incubated at 37°C for 24 h. An inoculum was made using the recently streaked *Salmonella* with 0.85% saline solution at a target absorbance reading of 0.12 OD that corresponds to approximately 10^8 cfu *Salmonella* /mL interpolated from a standard curve. The final absorbance reading for experiment 1 was 0.134 OD. Dilutions were made and duplicate nutrient agar (DIFCO, Becton, Dickinson and Co) plates were spread in order to confirm the concentration of the inoculum. The plates were incubated at incubated at 37°C for 24 h and then the colonies enumerated.

A total of 672 Cobb500 chicks (21/pen) were placed in 1.5 m x 1.2 m pens using a total of 32 pens, which included a tube-pan feeder (area= 0.28 m^2) and 7 nipple drinkers. For the first 3 d of brooding one additional feed tray was provided in each pen. Broilers were housed on 5 to 7.5 cm of new pine shaving litter in an environmentally controlled house at the University of Georgia Poultry Research Center. The broilers were housed according to standard brooding and growing conditions (Cobb500 Broiler Performance and Nutrient Supplement, Cobb-Vantress, Oct 2013). To detect any migrated chicks between adjacent pens, chicks in alternating pens were lightly sprayed with different color non-toxic food dyes to enable quick identification while

observed twice daily and any migrated chicks detected returned to the correct pen. Treatments were randomized and blocked on a pen basis with a total of 8 replicate pens for each of the 4 dietary treatments (see Appendix table 10). Day 0 weights were obtained and each chick selected to ensure that all chick weights were between 35 to 45 g, excluding the outlier chicks, so that each pen had a weight equal to the entire population in the experiment. Once all chicks were placed in the challenge room, 2 chicks were selected from each pen to be the seeders. Each seeder chick was confirmed by palpation to have an empty crop, marked on the head, wing banded in each wing and then orally inoculated with 0.2 mL of 1.95×10^7 cfu/mL *Salmonella* Typhimurium (approximately 3.9×10^6 cells each). Seeders were placed back in the pen in the feed tray to commingle with the penmates.

Feeding program. The feeding treatments in experiment 1 were as follows; basal control diet, 0.3% activated bamboo charcoal added (EcoBamboo), 0.6% activated bamboo charcoal added (EcoBamboo), and 0.12% Aromabiotic® a medium and short chain fatty acid product (NuScience, Drongen, Belgium). The control diet was used as the basal diet for all treatments was formulated on a digestible amino acid basis (NRC, 1994). Two levels of charcoal (3 g or 6 g/Kg) and one level of Aromabiotic® (12 g/Kg) were added as a part of the micro-ingredients mix that was added to the basal diet. Micro-ingredients and charcoal were premixed with an aliquant of corn prior to their addition to the mixer to assure a homogenous dispersion. The charcoal had been ground through a 0.2 mm sieve. The starter diet was fed from days 1 to 14, the grower diet was fed from days 14 to 28, and the finisher diet was fed from days 28 to 42. The composition of the control diets was based on a standard soybean commercial broiler diet.

Broiler weights. Broilers were weighted on 0, 14, 28, and 42 d of age on a per pen basis.

Chick Transport Basket Pads. To check for potential wild type Salmonellae, the paper pads from chick transport baskets (10 total) were folded and placed in individual 1-gallon zip-top plastic bags. Chick pads were transported to the USDA-RRC for *Salmonella* analysis. With clean latex gloves each pad was torn apart, placed in a new sterile plastic bag and 240 mL of 1% buffered peptone water (BPW, DIFCO) was added then incubated at 37°C for 24 h. Bags were squeezed in a pulsating motion for 30 sec and 0.5 mL of pre-enriched chick pad rinsate was added to 10 mL of Rappaport-Vassilidis (RV) broth (DIFCO) and 0.1 mL of pre-enriched rinsate was added to 10 mL of TT (Tetrathionate broth base, Hajna, Becton, Dickinson and Co. Sparks, MD). Tubes were then incubated at 42°C for 24 h. Following incubation, 20 µL from RV and TT broth were plated on XLT-4 (Hardy Diagnostics Criterion^{TM}, Santa Maria, CA) and Brilliant Green Agar with Sulfapyridine (Neogen, Lansing, MI) plates and incubated at 37°C for 24 h. All suspect colonies on XLT-4 (characterized as yellow/red colonies with black centers) or on BGS (pink colonies), were stabbed into Lysine Iron Agar (LIA) and Triple Sugar Iron (TSI) (Becton, Dickinson and Co) slant tubes, and incubated at 37°C for 24 h. All suspect positives were subjected to an agglutination test using Poly-O antigen (DIFCO; Becton Dickinson and Co. Sparks, MD) to confirm *Salmonella* from 6 serogroups (A, B, C_{1-3}, D, or E).

Drag Swab Sampling of Pen Litter. Litter drag swab samples were collected weekly from all pens. Drag swabs (n=1/pen, 7.62 × 7.62 cm, DS-001, Solar Biologicals Inc., Ogdensburg, NY) presoaked in skim milk were unwound and were dragged throughout the pen along the perimeter of the water line and feeder to maximize contact with areas that the chicks frequently defecate (Kingston, et al., 1981). The swabs were intermittently stepped-on at least 10 times and when present, feces were intentionally stepped-on (Buhr, et al., 2007).

Ceca Collection. The ceca of one seeder chick per pen was sampled at 1 wk and 2 wk were removed, euthanized and ceca collected to confirm *Salmonella* colonization. At 3 and 4 wk 2 penmate broilers, and 6 wk, a total of 5 penmate chicks per pen were randomly chosen, euthanized by electrocution and both ceca aseptically collected including the cecal tonsils and the ileal-cecal junction.

Salmonella Recovery. Drag swab samples were placed in sterile bags and 60 mL/swab of 1% BPW added. To obtain an average weight of the ceca collected each week, a random set of 5 samples of ceca or 5 breast skin (samples taken in Experiment 2) were weighed within plastic bags and the average of ceca and skin weights were calculated. Ceca were then macerated with a rubber mallet to ensure that the contents were exposed. To each bag 1% BPW was added mL at 3 times the g weight of the ceca. Drag swabs were manually mixed in a pulsating manner prior to streaking. Ceca and skins were mixed using a stomacher (Technar Company, Cincinnati, OH) for 1 min. Two 10 µL loops of solution were streaked onto BGS agar plates with nalidixic acid added at 200 µg/mL. Both the plates and samples were incubated at 37°C for 24 h. If direct plating was negative for *Salmonella*, plates were restreaked with another two 10 µL loops of the rinsate which had been enriched for 24 h and the plates again incubated at 37°C for 24 h. To confirm the presence of the marker *Salmonella* strain, representative suspect colonies were subjected to an agglutination test for the serogroup B (DIFCO) of which *Salmonella enterica* Typhimurium belongs. Samples that were positive from direct streaks were estimated to have $> 10^2$ cells/mL and samples that were positive only following enrichment, were estimated to have $< 10^2$ cells/mL in the initial sample dilution.

Materials and Methods Experiment 2

Salmonella Inoculum Animal Care and Housing . A total of 1,280 Cobb500 chicks (32/pen) were placed into 40 pens (the same room was cleaned out and new litter used in the same pens that were used in experiment 1). The inoculum was prepared as described for experiment 1 and the spectrometer OD reading for experiment 2 was 0.143 OD. As in experiment 1, chicks were weighed, selected, marked, and 2 chicks inoculated as seeders (0.2 mL of 1.4 x 10^8 cfu/mL *Salmonella* Typhimurium resulting approximately 2.9 x 10^7 cells per seeder chick) following the description for experiment 1. There were a total of 10 replicate pens for each dietary treatment. The treatments were as follows; basal control, 0.3% bamboo charcoal, 0.3% activated bamboo charcoal, and 0.3% pine charcoal (UGA, Bioconversion Research and Education Center). The bamboo charcoal came pre-ground while the pine charcoal was ground through a 0.2 mm sieve at the University of Georgia Poultry Research Center. All diets were formulated and mixed as described for experiment 1. Broilers were weighed by pen at day 0, 14, 28 and 42 d. However, the density of the pens changed by 3 wk due to broilers being removed weekly for ceca and pH sampling. Litter sampling by stepped-on drag swab occurred weekly as described in experiment 1. Ceca were collected as following the methods as described in experiment 1 at 1 and 2 wk, 1 seeder broiler chick per pen was euthanized and ceca were collected. At weeks 3, 4, 5, and 6, 5 penmate broilers per pen were euthanized and ceca collected. Explicit to Experiment 2, there was an outbreak of *Proteus* Mirabilis in the room, which affected reading litter and ceca results for *Salmonella*. Proteus on BGS with Nalidixic acid plates also turns the plates pink and can create similar colonies as to *Salmonella*. Proteus will swarm the plate, leaving it very difficult to isolate the marker *Salmonella* colonies. Agglutination of suspect colonies for *Salmonella* confirmation can be difficult because if there

are mixed colonies that are being picked out, the test can often create false positives (white chunks that can be mistaken to agglutination of the antigen to confirm Serogroup B). All samples that were confirmed positive with agglutination that had swarming of *Proteus* went through further confirmation. From each potential positive plate, 1 to 3 suspect colonies were each struck on to BGS-Nal plates for 3 consecutive days. Once colonies were isolated using CHROMagar *Salmonella* Plus plates (CHROMagar™ & Rambach™, Paris, France, www.CHROMagar.com, confirmed positive *Salmonella* with purple colonies) LIA and TSI slant tubes were also used to confirm *Salmonella*. All plates and slants were incubated at 37 °C for 24 h and the resulting suspect colonies went through an agglutination *Salmonella* confirmation test. A total of 40 samples that were not possible to isolate away from the *Proteus* went through Real-Time PCR (DuPont Bax® System PCR assays for *Salmonella*, Wilmington, DE) of which 4 were *Salmonella* positive.

pH recording. Of the broilers used for ceca sampling each week, 1 broiler/pen (40 sample/week; 10 broilers/treatment/wk) was selected for pH recording (typically the first broiler sampled for each pen). For 1 and 2 wk there was only 1 penmate broiler/pen sampled but from 3 to 6 wk, the first of the 5 penmate broilers/pen that was euthanized and ceca collected were sampled within 2 min. Once the first ceca from each pen was collected the duodenum and the crop was exposed and luminal pH de novo recorded using a pH probe (Hach model H280G, Loveland, CO). The duodenal loop was transected post flexure and the probe was inserted in the lumen (orad: towards the head). After reflecting the skin covering the crop a second probe was inserted in the lumen of the crop puncturing the crop wall directly with the probe to minimize any potential feed/liquid leaking from the crop. The probes remained in the lumen of the duodenum or crop for approximately 20 sec at which time the final pH reading was recorded.

After each reading, the electrodes were removed and cleaned using deionized water and placed into the pH 7 buffer until the next reading.

Breast Skin Collection after Defeathering. Twelve h prior to processing, broilers were individually banded and assigned a batch number that corresponded to the processing order, and there were 2 processing batches per feeding treatment on each of 2 consecutive processing days, resulting in a total of 4 batches (20 broilers) per feeding treatment. Each batch of broilers, 5 broilers per batch, were placed in a solid bottom coop for both feed and water withdrawal (Buhr et al., 2014). Processing took place at the USDA-ARS Russell Research Center pilot processing plant. Batches were processed one at a time and broilers were stunned at 20 volts for 10 sec, bled for 2 min, and soft-scalded by triple tank scalding at 52.8°C (127°F) for a total immersion time of 180 sec and then defeathered for 30 sec. Following defeathering, the entire rectangular breast skin sample which included the central sternal apterium, sternal feather tracts, and most of the adjacent pectoral apterium and pectoral feather tracts (Lucas and Stettenheim, 1972; Buhr et al., 2002) from each carcass was aseptically removed with sterile scalpel and forceps, storing scalpels and forceps in ethanol then flaming them before each additional collection. Breast skin was chosen as the sample tissue due to the ability to consistently remove all feathers and the cuticle layer of the skin during picking and because of the long-term contact between the breast and the environment during grow-out and transportation. Breast skin was placed in a labeled sterile plastic bag and stored on ice until the end of the collection, approximately 2 h. The picker and scalder surface-water were rinsed with 82°C water between each processed batch of broilers.

Salmonella Adsorptive test. This study was designed to test the ability of the three charcoals for *S*. Typhimurium adsorption within a liquid suspension. Approximately 3.4×10^7 cfu of nalidixic acid resistant *S*. Typhimurium as a 1 mL inoculum was added to 1 mL of brain-

heart infusion broth (DIFCO) and one of the three charcoals of varying concentrations (2, 6, 10, and 20 mg) were vortexed and then incubated at 37°C for 1 h with constant gentle agitation. These mixtures were then centrifuged at 30 RPM for 10 min to remove charcoal from the broth suspension. After centrifugation, 1 mL of the supernatant was diluted into 9 mL of sterile saline solution, vortexed and further diluted (10^{-3}, 10^{-4}, and 10^{-5}). A total of 100 µL of each dilution was plated (10^{-2}, 10^{-3}, and 10^{-4}) onto duplicate BGS with nalidixic acid (200 µg/L) plates, using 3 duplicate plates per concentration of each charcoal. Plates were incubated at 37°C for 24 h and the number of colonies enumerated. Plates that had colonies that were not well separated or if there were too many colonies to count (>300 cfu) were marked as TNTC (too numerous to count). Results are shown for only the 10^{-4} plates due to that the rest of the plates were read as TNTC.

Statistical Analysis. For weekly litter *Salmonella* recovery (positive or negative), a simple linear regression was used (SAS Institute, 1996) and slope compared among treatments. As seen in the results and discussion below, treatments between *Salmonella* recovery in the litter had a difference of only 1 to 3 pens (of 10 pens/treatment), and therefore no statistical tests were performed between treatments at any given week. Chi Square test for independence was used to detect differences in *Salmonella* recovery from the ceca within each treatment for each week of collection. For breast skin, the two combined days of processing and batches data were analyzed using the Chi Square test for independence for the recovery of *Salmonella*. A one-way ANOVA was used to determine differences (if any) of pH among the treatments during the week of collection as well as the overall differences in pH during the week of collection. Tukey's honestly significant difference procedure was used to separate the treatment means when an F-test was significant $P < 0.05$. To determine the significance of adsorptive properties of tested

charcoal, a one-way ANOVA was performed for statistical evaluation of the results. Tukey's honestly significant difference procedure was used to separate the amount of charcoal tested within the type of charcoal and the type of charcoal within a specific amount of charcoal used. Results are expressed as the arithmetic mean with the standard error of the mean (mean ± SD).

Results and Discussion

Litter Salmonella Recovery. Pen litter sampling results for experiment 1 are presented in Table 1 and Figure 1, and litter sampled for the first 2 wk was positive for *Salmonella* with direct plating (>10^2 cells/mL sample) in all pens except for a single pen for the 0.6% bamboo charcoal (7/8 pens were positive) at wk 1, however that pen was *Salmonella*-positive with enrichment and positive at wk 2. These results confirmed that the *Salmonella* challenge was successful for all pens in all feed treatment groups. At 3 wk, 1 wk after removing the remaining seeder at 14 d, *Salmonella* was not recovered from the litter via direct plating from 2 of the 0.6% bamboo charcoal pens and 1 of the Aromabiotic® pens sampled but was recovered from all but 1 of the 0.6% bamboo charcoal pens after enrichment. Over time, the recovery of *Salmonella* from the litter in the pens decreased significantly with direct plating on a weekly basis ($P < 0.05$) in all treatments comparing wk 1 had only 1 negative pen, wk 2 had 0 negative pens, wk 3 had 3 negative pens, then beginning at wk 4 the prevalence of *Salmonella* with direct plating rapidly decreased, with 7 negative pens, wk 5 had 21negative pens, and wk 6 had 31 negative pens of the 32 pens sampled (Figure 1), however there were no significant difference among the treatments. *Salmonella* prevalence began reducing by 3 wk thru 6 wk, although enrichment recovery did not drop until wk 6 as seen in Figure 2 and Table 1. *Salmonella* recovery among the treatments differed by only 1 or 2 of the 8 replicated pens with enrichment over all weeks of collection.

Therefore there were also no significant differences among treatments for the positive recovery of *Salmonella* by direct or with enrichment during any week. At 6 wk *Salmonella* recovery from the litter further decreases with 9 pens negative following enrichment plating.

During the summer months, unpublished data showed that by the end of a 6 wk grow-out, out of a total 21 pens, only 1 pen had recovery of *Salmonella* via direct plating and only 9/21 (43%) plates were positive with enrichment. In experiment 1 however, by 6 wk, 3/32 pens were positive via direct and 23/32 (72%) of the pens were positive with enrichment. At 8 wk (2 wk after the trial ended and no broilers remained in the pens), *Salmonella* was still present in 4 of the 32 pens. At 8 wk, the pens that had broilers fed Aromabiotic had no recovery of *Salmonella*, while charcoal and control pens had 3 or 4/8 positive pens with enrichment.

The sampling technique most used to sample the surface layer of litter in a poultry house is a drag swab (Bhatia et al., 1980; Kingston, 1981; Mallinson et al., 1989; Opara et al., 1992 and Caldwell et al., 1994). By intentionally stepping on the drag swab, which was done in the experiments, drag swabs are able to recover up to 64% more positive samples that otherwise would have been negative under conventional drag swab method (Buhr, 2007). When seeder chicks defecate on the litter and in the feed trays, penmates are able to consume *Salmonella* contaminated fecal material. Consumption of fecal covered litter and feed allowed *Salmonella* to be passed from seeders to penmates. Depending on litter type, broiler chicks have the potential to consume up to 6% of wood fiber during the first 7 d of life post placement. (Malone et al. 1983). Broilers can harbor *Salmonella* within the alimentary tract from hatch through grow-out although the number of birds shedding *Salmonella* declines over time. Day of hatch chicks have an essentially sterile gastrointestinal tract allowing *Salmonella* to colonize (Jayne-Williams et al. 1971) when consuming the seeder feces from the litter. Normal gut flora of the chick is

established within the first week and once established, causes the environment not to be condusive for *Salmonella*, decreasing the incidence of salmonellae colonization (Cox et al. 1990; Bailey 1987; Jayne-Williams et al. 1971). Because of this phenomenon, day-of-hatch chicks can become colonized by exposure to 100 times fewer *Salmonella* organisms than chicks challenged at Day 3 (Cox et al. 1990).

Results for litter *Salmonella* recovery for experiment 2 are presented in Table 2 and Figure 3 and the recovery of *Salmonella* had a similar trend as in experiment 1 where there was a significant decrease in the overall recovery with direct plating (only 7 of 40 positive at wk 6) and there were no significant differences among the treatments at each week sampled. For the first 3 wk of experiment 2, all of the pens were positive via direct plating except for a single pen for the bamboo charcoal at wk 1 and a single pen for the activated bamboo charcoal at wk 2, but both were positive following enrichment. By 4 wk, there was a significant decrease ($P < 0.05$) in recovery with direct plating, but similar to experiment 1 there were no differences among treatments. From 3 wk when 100% of the pens were *Salmonella* positive to 4 wk only 25% of the pens remained positive, although all pens were positive following enrichment. Similar to experiment 1, *Salmonella* peaked in prevalence at 3 wk and *Salmonella* in the litter became harder to detect with direct plating. With direct plating, at wk 1 there was 1 negative pen, wk 2 had 1 negative pen, week 3 had 0 negative pens, wk 4 had 30 negative pens, wk 5 had 26 negative pens, and wk 6 had 33 negative pens out of the 40 pens. However, all of the plates that were positive via direct only had 1 to 5 colonies from the 100 µL aliquot. This indicates that with direct plating (>100 cells/mL of sample), that the recovery was between 100-500 cells/mL in 6/10 pens. At 6 wk, the bamboo charcoal group was again 2/10 pens being positive with direct plating. At 6 wk, the recovery of *Salmonella* via direct plating was the lowest at only 1 to

3/10 pens. Recovery with enrichment also had a decreasing trend from 100% positive recovery at 5 wk to only 7 or 8/10 pens positive recovery with enrichment (29/40 pens). In experiment 2, pens had *Salmonella* in all treatments and all pens with enrichment up until 6 wk, where the low recovery was in the control and bamboo charcoal treatments with 7/10 positive.

The recovery of *Salmonella* in all pens remained about the same throughout the experiment, with a maximum difference of up to 3/10 pens. None of the charcoals, unlike Aromabiotic, had any detectable effect on the litter at the end of the study in experiment 1. Adding charcoal in both experiment 1 and experiment 2 show no significant difference in the recovery of *Salmonella* from the litter compared to the controls. Charcoal had been fed for adsorption of aflatoxins that were contained in defecated feces (Hatch et al., 1984, Dalvi et al., 1984).

Ceca Salmonella recovery. In experiment 1, ceca were collected from penmates at wk 3, 4, and 6 for all treatments (Control, 0.3% Charcoal, 0.6% Charcoal and Aromabiotic). All seeder chicks at both 1 and 2 wk were confirmed to have been colonization by *Salmonella* (data not shown). Results presented in Table 3, show at 3 wk both 0.3% and 0.6% activated bamboo charcoal treatments had significantly lower (7/16 ceca) recovery of *Salmonella* from ceca via direct plating compared to the control group (13/16 ceca; $P < 0.05$), but were not significantly different following enriched plating (14 to 16/16 ceca). Similar to the litter results of both experiments 1 and 2, by 3 wk, the time to confirm colonization of *Salmonella*, both charcoal groups were shown to have lower colonization at a level as recovered with direct plating ($>10^2$ cells). Charcoal by wk 3 may be reducing colonization by quantity, though not qualitatively since recovery of *Salmonella* was not different between treatments following enrichment. By 4 wk, the overall recovery of *Salmonella* had decreased and Aromabiotic supplemented feed had

the highest recovery from direct plating at 38%. Although not significantly different, the highest recovery of *Salmonella* with enrichment of cecal samples was in the control group at 85% while the recovery from 0.3% charcoal 64%, 0.6% charcoal 54%, and Aromabiotic 57%. At 6 wk, the recovery of *Salmonella* was lower in all treatments and the control group had a recovery of 33% (13/40). Aromabiotic had a significantly lower recovery from ceca of *Salmonella* at 15% (6/40) compared to the control ($P < 0.05$). The 0.3% charcoal approached significance (7/40 ceca; $P = 0.064$) *Salmonella* positive compared to the control group, however the 0.6% charcoal was at 23% *Salmonella* positive (9/40 ceca) and did not differ from the control.

Ceca in experiment 2 were sampled for *Salmonella* on a weekly basis from all 48 pens for all treatments (Control, bamboo charcoal, activated bamboo charcoal and pine charcoal). At 1 and 2 wk, colonization of *Salmonella* in all seeder chicks was confirmed and all penmate ceca (1 chick/pen) at 1 and 2 wk were also all positive with direct plating (data not presented), confirming that with 1 wk seeder exposure, penmates were colonized with *Salmonella*. At 3 wk, the colonization of *Salmonella* of the penmates in each treatment was not significantly different (60 to 70% positive, Table 4). Enrichment plating at wk 3 was not done due to the presence of the bacteria *Proteus mirabilis*, a gram-negative facultative anaerobe which also ferments H_2S and changed the BGS plates pink and resulting in similar looking colonies to *Salmonella*, took over the remaining plates and samples were discarded before further analysis could be obtained. Additional plating of suspect colonies on different isolation media (Chromagar and XLT-4 plates, LIA and TSI slants,) as well as PCR for select colonies to confirm *Salmonella*, took place in experiment 2 that did not occur in experiment 1.

Similar to experiment 1, the recovery of *Salmonella* in the ceca was lowered each sequential week from wk 4 to 6. At 4 wk, only the activated bamboo charcoal, had a

significantly lower recovery of *Salmonella* 35/50 (70%) compared to the control 44/50 (88%). However at 5 wk, no treatment group was significantly different from one another due to the overall decline in *Salmonella* with age. At wk 5 *Salmonella* cecal recovery from direct plating ranged from 36 to 46% and following enrichment from 68 to 82%. At 6 wk cecal *Salmonella* recovery from direct plates ranged from 30% for the control to18% for both the activated bamboo charcoal and for pine charcoal and 26% for the bamboo charcoal. Following enrichment at wk 6 cecal *Salmonella* recovery ranged from 48% for pine charcoal to 56 for activated charcoal and 58% for bamboo charcoal and 62% for the control and did not differ significantly among all treatment groups.

pH of the crop and duodenum. Only in experiment 2 was the pH of the crop and duodenum sampled weekly in full fed broilers within 2 minutes following euthanasia. There were no significant differences in pH between treatments in any given week for the crop and duodenum. However, in the crop the overall pH among wk 1 was significantly higher than wk 2 through 6 ($P < 0.05$). Figure 5 shows the significant drop from wk 1 throughout the rest of grow-out for the four treatments (n=10). The average pH at wk 1 was 5.96 (n=40). After wk 1, pH began to decrease, wk 2 4.35; wk 3 4.71; wk 4 4.74; wk 5 5.09; and wk 6 4.99 (n=40). The pH at 1 wk was close to neutral which could enable *Salmonella* from the seeders to colonize the alimentary tract of penmates. Opening the crop after the pH was recorded, there was very little to no feed in the crop. By 7 d, penmate chicks in this experiment had not consumed enough feed to completely fill their crop. Empty crops, similar to broilers going through feed withdrawal, the pH rises toward neutral. There is less fermentable carbohydrates from feed for lactic acid bacteria such as Lactobacilli to thrive, resulting in a rising crop pH (Fuller, 1977). The crops from 6 wk old broilers can increase by 1.0 (5.5 to 6.5) pH unit within 6 to 24 h without feed

(Hinton et al., 2000) and the prevalence of *Salmonella* in the crop increases from 12% (with feed) to 88% (without feed at18 h) for broilers challenged with *Salmonella* prior to feed withdrawal (Ramirez et al., 1997). Though not exactly the same situation, *Salmonella* colonization rate increases when crops are empty before broilers, broiler breeders, or laying hens are orally challenged, because the higher pH of the crop enables *Salmonella* colonization.

The age of poultry at the time of exposure to *Salmonella* plays a critical role to colonization. Milner and Shaffer (1953) found that day old chicks could be colonized with less than 5 cells of *Salmonella*. However if challenging 50 wk broiler breeders with 2.5 $x10^8$ cells of *Salmonella* Enteritidis, when feed withdrawn for 24 h, 7 days post challenge only 8/20 were positive in the ceca with enriched plating (unpublished data, 2013) .

Duodenum pH among all treatments at wk 1, 2, and 6 was significantly higher than at wk 3, 4, and 5 ($P < 0.05$). Wk 1 overall pH was 6.03; wk 2 6.02; wk 3 5.85; wk 4 5.86; wk 5 5.86; and wk 6 5.94. Duodenal pH values in this study were not different from those found throughout the literature (Zou et al., 2009; Walk et al., 2012; Morgan et al., 2014). The pH remained close to neutral. Beginning at wk 3, broilers begin to exclude *Salmonella* as was seen in experiment 1throughout literature poultry over time will naturally exclude *Salmonella* (Gustafson and Kobland 1984). The lowered pH may assist with the lower prevalence and recovery of *Salmonella*.

Although *Salmonella* normally do not survive at a pH 3.0 in culture, however, Kwon and Ricke (1998) found that a higher number of bacteria survived for several hours after exposure to any volatile fatty acid in-vitro. This suggests that *Salmonella* could be protected against a lower pH environment, in this case, *Salmonella* could be protected against gastric acid when passing through the gastrointestinal tract. *Salmonella* was able to pass through the proventriculous and

gizzard which have pH as low as 2 to 2.5, into the crop where at the first week was close to neutral (high value of 6.44) and the duodenum (6.52) to its primary *Salmonella* colonizing site, the ceca. Kwon and Ricke (1998) suggested that the protection is caused by the expression of genes involved in an acid-tolerance response and the synthesis of a series of acid shock proteins that are protective against extreme acidic conditions.

Using a pH probe and inserting it directly into the lumen of the crop and duodenum to obtain pH was stated by Morgan et al. (2014) to be representative of bird gastrointestinal environment because it encourages dissociation of carbonic acid, the major buffer in the gastrointestinal tract, which causes the pH to read higher than when measured in situ. Their study evaluated the gizzard and duodenum and the influence of additional dietary calcium (using high or low level or limestone) level on pH. The pH of the duodenum for 7 and 28 d (5.89 and 5.93) was significantly different from 14 d (6.14). Their feeding treatments did not affect the pH of the duodenum. The pH differences are similar to what was seen in experiment 2 pH duodenum.

***Breast skin Salmonella* recovery.** Only in experiment 2 was breast skin and the corresponding transport and feed withdrawal coops sampled for *Salmonella*. The results presented in Table 5, on the first day of processing (d 43), the control (*Salmonella* positive) group was the only group to have recovery of *Salmonella* from breast skin following enrichment. For both positive control batches, the transport coop floors were *Salmonella* positive. Breast skin from positive control batches were positive (3/10) indicating that *Salmonella* was not completely removed during soft scalded and defeathered. In the bamboo charcoal and the pine charcoal treatments, there were positive coops (1 or 2/2 positive, respectively). For all three charcoal treatments *Salmonella* was not recovered from breast skin, indicating that even when *Salmonella*

was present on the coop floors (from feces) the *Salmonella* was removed during scalding and defeathering below the level of detection on the breast skin. The outside of the broiler (feathers, skin, and feet) becomes contaminated with feces during grow-out. During cooping, transport, and holding broilers increased the concentration of feces on the broiler carcass (Buhr et al., 2000). Solid bottom coops allowed fecal material to accumulate and contaminate the outside of the broilers during the 12 h prior to processing. Soft scalding (127ºF/53ºC for 180s), scalding at a lower temperature for twice as long compared to hard scalding (140ºF/60ºC for 90s) allowed for the detection of *Salmonella* on the breast skin (unpublished data).

On the second processing day (d 44), the control group had the highest overall recovery of *Salmonella* on the breast skin and both batch coop floors were also positive. One of the 2 batch coop floors were *Salmonella* positive for all 3 charcoal treatments. However, coop floors from bamboo charcoal (batch 2), pine charcoal (batch 8) and from negative control (batch 10) were found to be negative but their breast skins were positive (2 to 5/10 carcasses sampled). Breast skin *Salmonella* recovery by processing batch is presented in Table 7, batch 2 and batch 8 could have been contaminated from residual *Salmonella* left in the picker from the previous batches 1 and 7, respectively, since those batches had positive recovery of *Salmonella.* However for batch 10, the previous 2 batches picked did not have *Salmonella* recovery from any of the 10 previously process carcasses. Both the picker and all 3 scalders (surface foam) were sprayed with water at 82°C between each processing batch. The occurrence of *Salmonella* positive breast skin on the first and last carcass in the negative control batch (for both only a single *Salmonella* colony) may be attributable to residual *Salmonella* in the picker that was not removed by hot water rinsing. The 2 d cumulative of breast skin *Salmonella* results are show in Table 6 that all

treatment groups had significantly lower (0 to 15% positive; $P < 0.05$) recovery of *Salmonella* compared to the control feed group at 40% positive.

Though the ceca of all treatments were not different (except wk 4 for activated charcoal 35/50 vs. 44/50 positive for the control), breast skin had significantly lower detected *Salmonella*. *Salmonella* recovery at 42 d 46% with enrichment was at the lowest percentage, yet the breast skin recovered up to 80% showing that ceca recovery should not be used as a reliable predictor of breast skin recovery of *Salmonella*.

Adsorption properties. Table 8 shows that and activated bamboo charcoal increased adsorption of *Salmonella* Typhimurium (lower $\log_{10}$cfu recovered on the plates) with increasing amounts of charcoal. Tubes that had 2 mg of each of the charcoals resulted in higher $\log_{10}$cfu of *Salmonella* recovery compared to when 20 mg of charcoal was added. The difference from 2 mg of activated bamboo charcoal to 20 mg was 0.5 $\log_{10}$cfu. Though significantly, if these samples were collected in-vivo (litter, ceca, breast skin, etc), all samples would have been positive with direct plating, since detection is at approximately $<10^2$ cells. The inoculum level maximum that could have been recovered onto the plates read was at 2.5 log10 (3.4×10^2 cfu). All plates read in Table7 had less than 2.5 log of *Salmonella* Typhimurium. Therefore, the log reduction of having no charcoal versus having charcoal in-vitro was at 2.5 log (20 mg bamboo charcoal) and the second highest was 1.2 log reduction (10 mg activated bamboo charcoal), an overall 1 to 2 log reduction. Within charcoal types, only at 10 and 20 mg of charcoal was noted to have a significant difference, activated bamboo charcoal had the lowest recovery at log 1.4 then bamboo charcoal 2 then pine charcoal at 2.3 at 10 mg. At 20 mg, bamboo charcoal had no recovery of *Salmonella*, activated bamboo charcoal had a log 2 and pine charcoal at 2.4.

Previous work has indicated that activated charcoal has a high adsorptive capacity although it tends to be nonselective (Wicks et al., 1980; Chandy and Sharma, 1998). However, adsorptive capacity of charcoal may depend on the pore size as well as whether or not it is activated, which is also related to pore size. For removal of relatively large materials activated charcoal with large pores is needed, whereas small substances require small pores (Chandy and Sharma, 1998). In this study, *Salmonella* Typhimurium was more effectively adsorbed by bamboo charcoal, which was not activated, compared to activated bamboo charcoal and pine charcoal. The differences were only seen if there was a higher amount (10 or 20 mg) of charcoal in the tubes. However the origin of charcoal seems to make a difference, pine charcoal did not adsorb as much *Salmonella* Typhimurium compared to either of the bamboo charcoals. The probable reason why bamboo charcoal has higher binding capacity to *S.* Typhimurium may be due to smaller pore size is more ideal to capture *Salmonella*.

Performance Study Materials and Methods

Feed preparation. A performance study was conducted at the same time as experiment 2 but in an adjacent animal room and without a *Salmonella* challenge. Therefore, feed preparation was the same as for experiment 2 described previously and the treatments were also the same as in experiment 2; basal control, 0.3% bamboo charcoal, 0.3% activated bamboo charcoal and 0.3% pine charcoal. The starter, grower, and finisher diets for all treatments were made from a common basal diet (control), Table 10 in the appendix. Feed for each treatment was weighed by pen at placement.

Animal Care and Housing. The same housing environment settings were used in the performance study as were used for experiments 1 and 2 except for the litter. Litter in the

performance study was used from 2 previous broiler flocks. A total of 1,008 male Cobb500 chicks (21 chicks/pen) were placed into 48 pens. The initial weight of each chick was between 40 to 50 g. Treatments were randomized and blocked on a pen basis with a total of 12 replicate pens for each of the 4 dietary treatments.

Body Weight and Feed Conversion. To determine performance in the absence of *Salmonella* challenge, broilers were weighed at 0, 14, 28, and 42 d on a per pen basis. Day 0 weights were obtained by weighing chicks into groups of 21 so that each pen had a weight that was equal to the entire population of the study. Pen weights were approximately between 897 to 918 grams (42.7 to 43.7 grams/chick). Remaining feed was also weighed on a per pen basis at 14, 28, and 42 d. All coops were pre-weighed before any broilers were weighed, and coop weights were subtracted in order to obtain actual weight of the broilers in each coop. At the end of grow-out, 42 d, individual broilers within each pen were weighed enabling individual bird weights and the calculation of total pen weight.

Feed was weighed at the beginning of each phase and weighed back at the end of each phase, starter (0 to 14 d), grower (14 to 28 d), and finisher (28 to 42 d). At the end of the experiment any feed that was remaining in the feeders was weighed and subtracted from the total added. The feed conversion, mortality-corrected feed conversion was calculated based from the data. Mortality and culling records including weight and the cause of death were kept daily for each pen. A cull was defined as a bird that could not access the water line. Beginning at 0 d, the weight (in grams) of all mortality was recorded. The date and weight of all mortality and culls was used to adjust the feed conversion per pen.

The *Salmonella* challenge study experiment 2 broilers were also weighed at 0, 14, 28 and 42 d the same as described for the performance study. However in the *Salmonella* challenge 2

room, disposable boot covers and gloves were changed between pens so to not transfer *Salmonella* from one pen to another. Also, in the *Salmonella* challenge experiment 2 room bird density decreased weekly throughout the experiment due to the weekly removal of 5 broilers/pen for ceca collection. The weekly decreasing bird density was the main reason for the additional performance study to accompany experiment 2.

Statistical Analysis. Performance data were subjected to ANOVA according to the General Linear Model (GLM) and statistics were completed with the Minitab statistical software package (Release 16, State College, PA).

Performance Study Results and Discussion

Body weight, body weight gain, and the feed to gain ratio from 0 to 14, 14 to 28, 28 to 42 and 1 to 42 d did not differ significantly ($P > 0.05$) among the broilers fed any of the 4 dietary treatments (Table 8). The body weights of broilers weighed at 14 d ranged approximately from 0.42 and 0.43 kg. The body weights of broilers weighed at 28 d ranged from about 1.59 to 1.62 kg. The final live body weights of broilers weighed at 42 d ranged from about 3.08 to 3.14 kg. Broilers were below the standard curve for Cobb-Vantress Cobb500 weights at 14 d but above the standard weights at 28 d and 42 d. Correspondingly, the weight gained for the first 14 d in all treatments was below the standard curve for Cobb500 broilers, however the weight gained during the grower and finisher phase was above the standard Cobb500 weight gained. The feed to gain ratio was lower in the performance study birds at 0 to 42 d at 1.54 kg compared to the standard Cobb500 feed to gain ratio of 1.70 kg. The feed to gain ratio was lower at 1.24 during the starter phase (0 to 14 d) compared to the standard Cobb500 of 1.36 lower 1.36 in the grower phase (14 to 28 d), and lower at 1.77 in the finisher phase (28 to 42 d) compared to the standard

Cobb500. In experiment 2, for the *Salmonella* challenged broilers fed a diet supplemented with the activated bamboo charcoal (0.43 and 1.59 Kg) weighed more than the basal control (0.42 and 1.56 Kg) birds at 14 and 28 d of age, respectively (Table 9). These results suggest that when broilers were challenged with *Salmonella* and placed on new litter adding activated bamboo charcoal to the feed had a marginal effect on growth. However for broilers that were not challenged and placed on used litter, activated bamboo charcoal has no detectable effect on growth performance. The performance study and the *Salmonella* challenge study of experiment 2 cannot be directly compared to one another due to bird density changing dramatically on a weekly basis in the experiment 2 *Salmonella* challenge room for all pens (5 broilers/wk sampled for pH and ceca). During ceca collections in the experiment 2 room, smaller birds were first chosen for collections to minimize culling, leaving bigger birds in the pens on a weekly basis. The temperatures and relative humidity of the rooms, though having the same protocol, were not observed to exactly the same in both rooms on a day-by-day basis.

CHAPTER 4

CONCLUSIONS

Overall, between the two experiments charcoal had a minimal effect on *Salmonella* prevalence, though some minimal differences occurred during the grower diet phase (wk 3 in experiment 1 and wk 4 in experiment 2) which is the time where broilers begin to eliminate *Salmonella* from the alimentary tract. Litter *Salmonella* recovery was also not different between charcoal treatments, though similar to ceca, the litter also decreased in prevalence of *Salmonella* over time. The pH of the crop and duodenum were not changed due to the additional charcoal at any week sampled. However, the detection of near pH neutral conditions in the crop during the first wk may explain the possibility for *Salmonella* to colonize and spread to the penmates within 1 wk. Further research is needed to determine in acidic prebiotics that can lower the pH of broilers in a larger scale within the first 7 d.

Dietary charcoal had an effect on the recovery of *Salmonella* at the end of grow-out on the broiler carcass post defeathering. Adsorption of *Salmonella* was achieved using charcoal in-vitro which may not answer the questions of the cecal recovery but may have a correlation with *Salmonella* recovery on the outside of the broiler.

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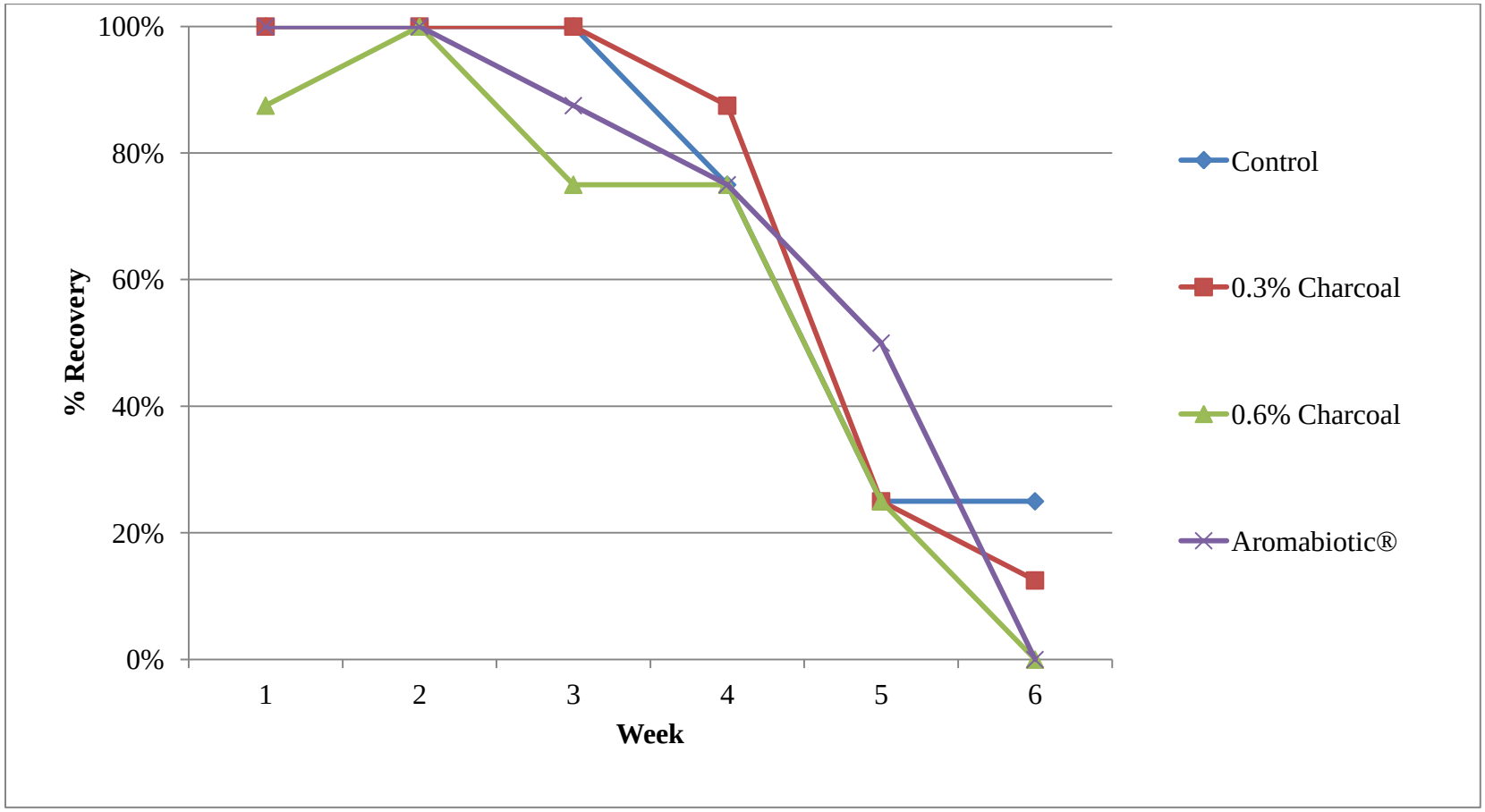

Figure 1. *Salmonella* Typhimurium recovery from the litter via direct plating performed on a weekly basis, Experiment 1. Observed *Salmonella* litter recovery reduced after 3 wk in all treatments but no significant treatment effect detected ($P > 0.05$, n=8).

Note: For all treatments *Salmonella* recovery decreased significantly from week 3 to 6, though not significantly different from one another

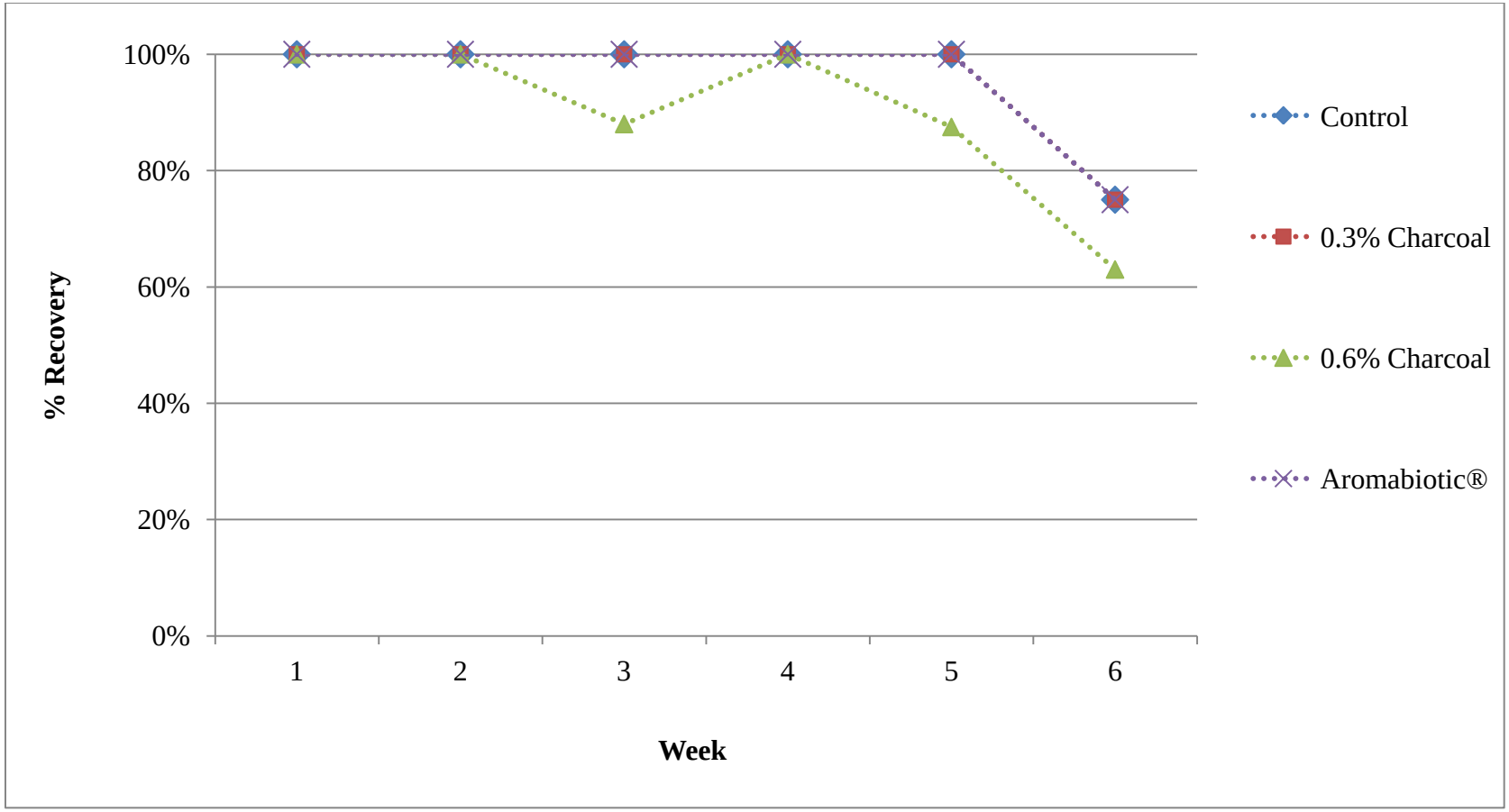

Figure 2. *Salmonella* Typhimurium recovery from the litter via enriched plating performed on a weekly basis, Experiment 1.

Note: Observed *Salmonella* litter recovery reduced after 3 wk in all treatments but no significant treatment effect detected

($P > 0.05$, n=8)

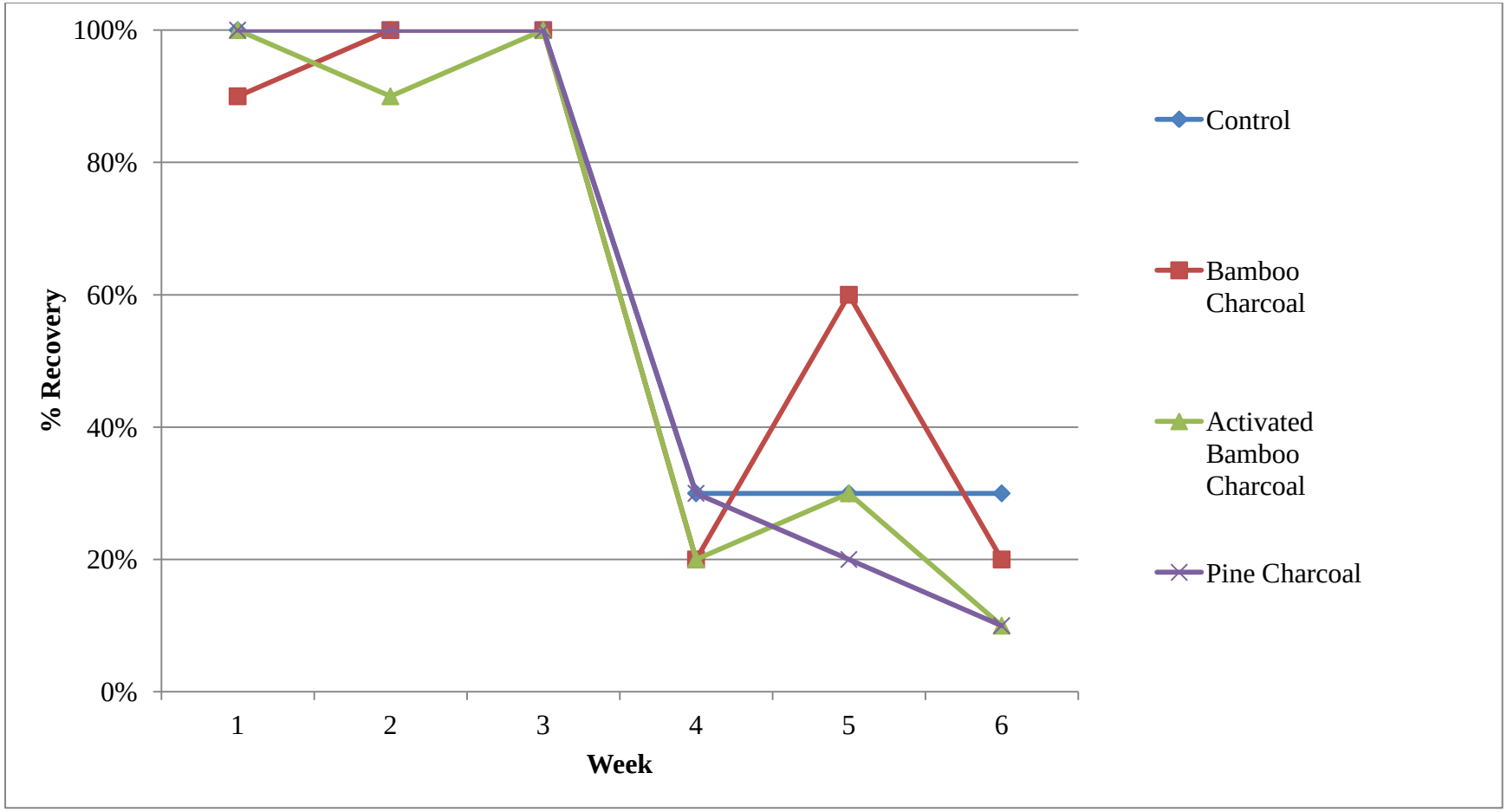

Figure 3. *Salmonella* Typhimurium recovery from the litter via direct plating performed on a weekly basis, Experiment 2. Observed *Salmonella* litter recovery reduced after 3 wk in all treatments but no significant treatment effect detected ($P > 0.05$, n=10).

Note: For all treatments *Salmonella* recovery decreased significantly from week 3 to 6, though not significantly different from one another

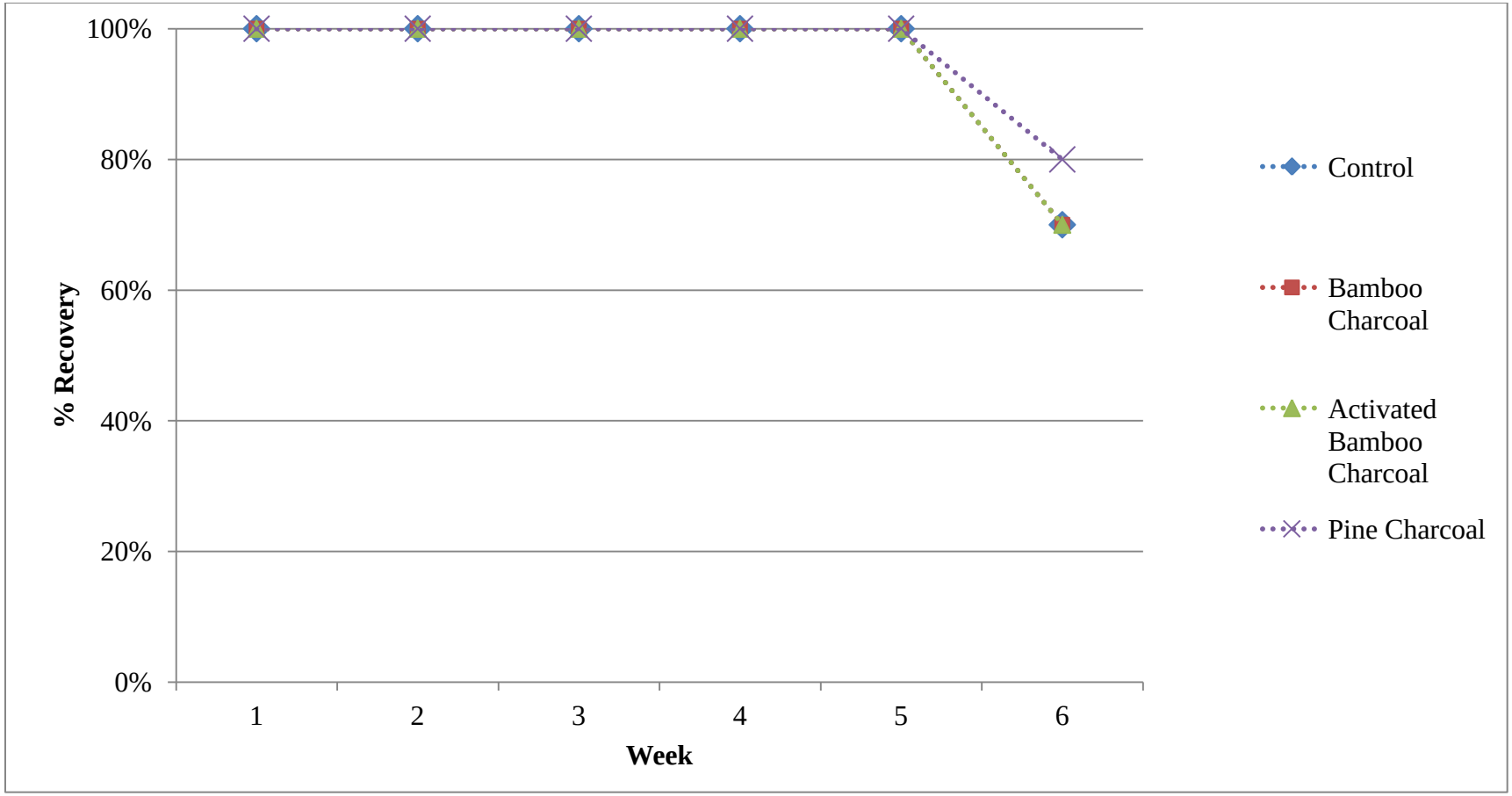

Figure 4. *Salmonella* Typhimurium recovery from the litter via enriched plating performed on a weekly basis, Experiment 2.

Note: Observed *Salmonella* litter recovery reduced after 3 wk in all treatments but no significant treatment effect detected ($P > 0.05$, n=10)

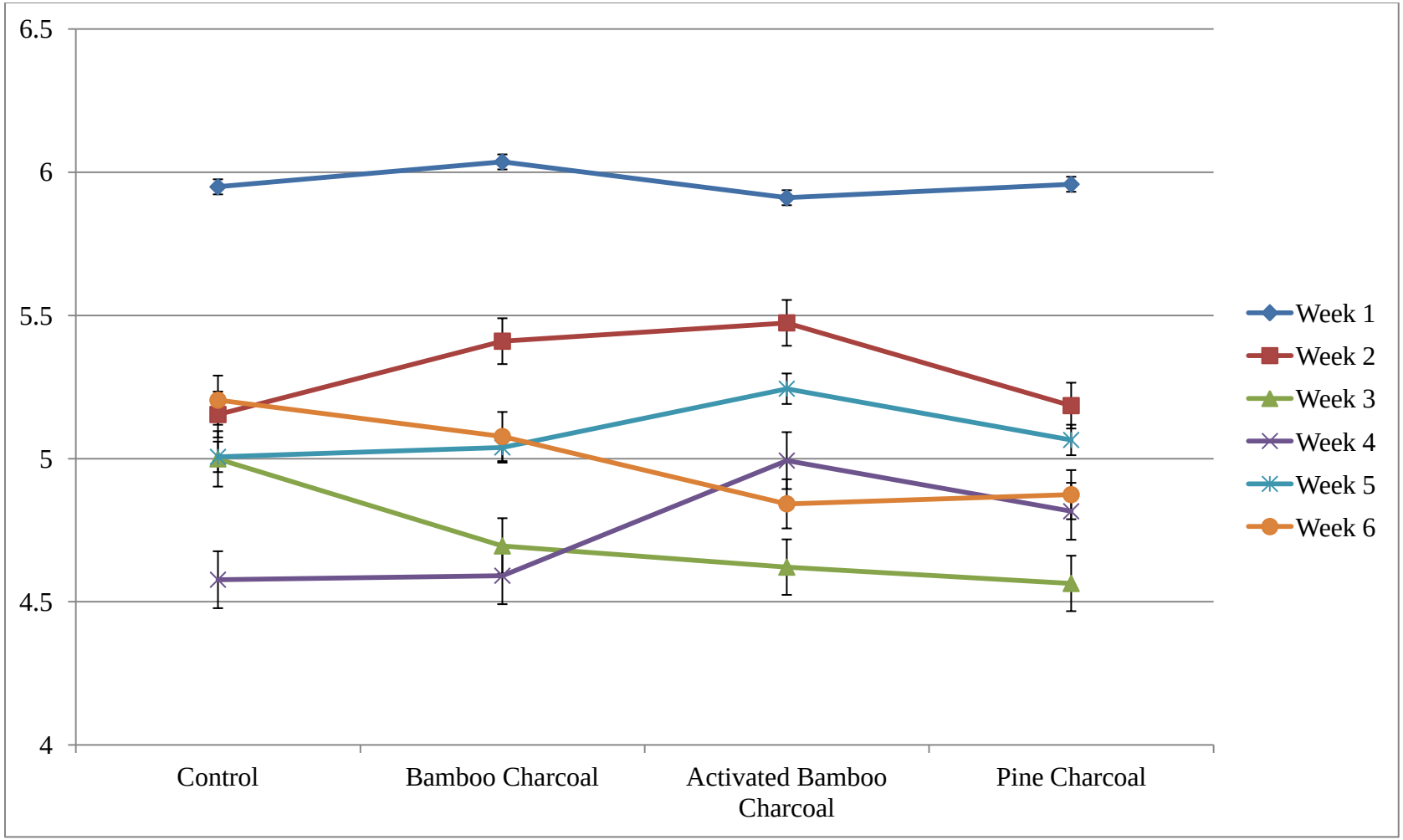

Figure 5. Weekly crop pH with standard error bars using de novo technique.

Note: All treatments had a significantly higher pH in week 1 compared to week 2, 3, 4, 5, and 6 ($p < 0.05$) There was no significant difference among treatments at any given week, Experiment 2, (n=40)

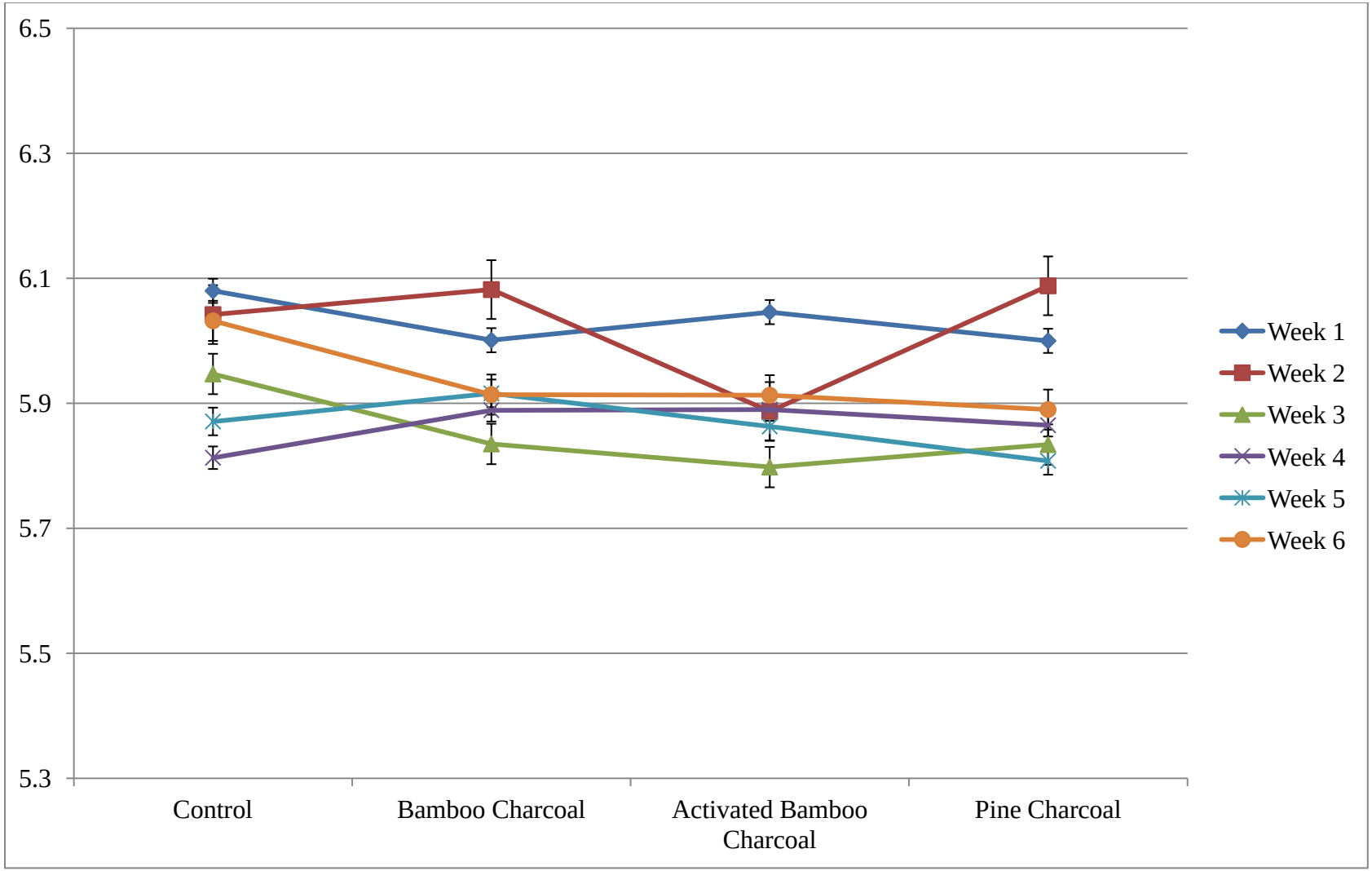

Figure 6. Weekly duodenum pH with standard error bars using de novo technique.

Note: There was no significant difference among treatments at any given week, Experiment 2, (n=40)

Table 1. *Salmonella* Typhimurium recovery from litter samples from broilers exposed to seeders challenged with *Salmonella* on the day of placement thru week 2, experiment 1

	Salmonella Recovery											
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
Treatments[1]	Direct[2]	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched
Control	8/8	8/8	8/8	8/8	8/8	8/8	6/8	8/8	2/8	8/8	2/8	6/8
0.3% Bamboo charcoal	8/8	8/8	8/8	8/8	8/8	8/8	7/8	8/8	2/8	8/8	1/8	6/8
0.6% Bamboo charcoal	7/8	8/8	8/8	8/8	6/8	7/8	6/8	8/8	2/8	7/8	0/8	5/8
0.6% Aromabiotic®	8/8	8/8	8/8	8/8	7/8	8/8	6/8	8/8	4/8	8/8	0/8	6/8

[1] Feed additive administered in feed beginning day of placement in starter, d 14 grower and d 28 finisher.

[2] D=Direct positive plating (>10^2 cells/mL); E=Enriched positive plating (<10^2 cells/mL).

[3] Number positive/ total number of pens sampled, 32 pens total.

No significant differences were detected among treatments within any week sampled, $P > 0.05$.

Table 2. *Salmonella* Typhimurium recovery from litter samples from broilers exposed to seeders challenged with *Salmonella* on day of placement thru week 2, experiment 2

	Salmonella Recovery											
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
Treatments[1]	Direct[2]	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched
Control	10/10[3]	10/10	10/10	10/10	10/10	10/10	3/10	10/10	3/10	10/10	3/10	7/10
0.3% bamboo Charcoal	9/10	10/10	10/10	10/10	10/10	10/10	2/10	10/10	6/10	10/10	2/10	7/10
0.3% Activated bamboo Charcoal	10/10	10/10	9/10	10/10	10/10	10/10	2/10	10/10	3/10	10/10	1/10	7/10
0.3% Pine Charcoal	10/10	10/10	10/10	10/10	10/10	10/10	3/10	10/10	2/10	10/10	1/10	8/10

[1] Feed additive administered in feed beginning day of placement.

[2] D = Direct positive plating (>10^2 cells/mL); E = Enriched positive plating (<10^2 cells/mL).

[3] Number positive/ total number of pens sampled, 40 pens total.

*The number of positive pens with direct plating decreased significantly in all treatments by 4 wk; no differences detected among treatments within any week, $P > 0.05$.

Table 3. Percentage positive *Salmonella* Typhimurium cecal recovery from broilers exposed to seeders challenged with *Salmonella* day of placement

	Salmonella Recovery					
	Week 3		Week 4		Week 6	
Treatment[1]	Direct[2]	Enriched[2]	Direct	Enriched	Direct	Enriched
Control	13/16 (81)	16/16 (100)	3/13 (23)	11/13 (85)	3/40 (8)	13/40 (33)
0.3% Activated bamboo charcoal	7/16 (44)*	14/16 (88)	2/14 (14)	9/14 (64)	2/40 (5)	7/40 (18)
0.6% Activated bamboo charcoal	7/16 (44)*	14/16 (88)	3/13 (23)	7/13 (54)	2/40 (5)	9/40 (23)
0.12% Aromabiotic®	12/16 (75)	15/16 (94)	6/16 (38)	9/16 (57)	0/40 (0)	6/40 (15)*

[1] Feed additive administered in feed beginning day of placement.

[2] Direct positive plating (>10^2 cells/mL); Enriched positive plating includes direct plating (<10^2 cells/mL).

[3] Number positive/number ceca sampled (percentage positive).

* Significantly different from control treatment of the week collected, $P < 0.05$.

Table 4. Percent positive *Salmonella* Typhimurium cecal recovery from broilers exposed to seeders challenged with *Salmonella* day of placement

	Salmonella Recovery							
	Week 3		Week 4		Week 5		Week 6	
Treatment[1]	Direct[2]	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched
Control	35/50[3] (70)	ND[4]	22/50 (44)	44/50 (88)	23/50 (46)	37/50 (74)	15/50 (30)	31/50 (62)
0.3% Bamboo charcoal	34/50 (68)	ND	24/50 (48)	38/50 (76)	17/50 (34)	34/50 (68)	13/50 (26)	29/50 (58)
0.3% Activated bamboo charcoal	35/50 (70)	ND	19/50 (38)	35/50 (70)*	23/50 (46)	33/50 (66)	9/50 (18)	28/50 (46)
0.3% Pine charcoal	30/50 (60)	ND	29/50 (58)	43/50 (86)	28/50 (36)	41/50 (82)	9/50 (18)	24/50 (48)

[1] Feed additive administered in feed beginning day of placement.

[2] Direct positive plating (>10^2 cells/mL); Enriched positive plating includes direct plating (<10^2 cells/mL).

[3] Number positive/number ceca sampled, 200 ceca collected per week.

[4] ND: Not Done.

*Significantly different from control treatment $P < 0.05$.

[ł]Penmates at 1 and 2 wk were all positive with direct plating (>10^2cells).

Table 5: Percentage positive *Salmonella* Typhimurium breast skin post-pick and the coops that were used for transportation and holding from broilers exposed to seeders challenged with *Salmonella* Typhimurium at day of hatch

	Salmonella Recovery					
Treatment	Skin 43 d	Coop 43 d	Skin 44 d	Coop 44 d	Total Skin	Total Coop
Control	3/10[3]	2/2	5/10	2/2	8/20 (40)	4/4
0.3% Bamboo charcoal	0/10	2/2	2/10	1/2	2/20 (10)*	3/4
0.3% Activated bamboo charcoal	0/10	0/2	3/10	1/2	3/20 (15)*	1/4
0.3% Pine charcoal	0/10	1/2	0/10	1/2	0/20 (0)*	2/4
Negative control	0/8	0/2	2/8	0/2	2/16 (13)*	0/4

[1] Feed additive given to broilers starting day of placement.

[2]Negative control = broilers that were not challenged with *Salmonella*.

[3]Number enriched positive/number breast skin samples, 44 breast skin samples per day.

*Significantly different from control treatment $P < 0.05$.

Table 6: The recovery of *Salmonella* Typhimurium from the coops that held broilers that went through feed withdrawal for 12 h and were processed on two consecutive days

Batch	Treatment	43 d	44 d
1	Control	+	+
2	0.3% bamboo charcoal	+	-
3	0.3% activated bamboo charcoal	-	+
4	0.3% pine charcoal	-	+
5	Negative control	-	-
6	Control	+	+
7	0.3% low grade bamboo charcoal	-	+
8	0.3% activated bamboo charcoal	+	-
9	0.3% pine charcoal	+	-
10	Negative control	-	-

*Negative control (broilers not challenged with *Salmonella* Typhimurium.

*Broilers were processed in numerical batch order.

*+: positive; -: negative.

*All recovery was positive with enrichment ($<10^2$ cells/mL).

Table 7: *Salmonella* Typhimurium recovery $\log_{10}$ (colony forming units) in-vitro with bamboo charcoal, activated bamboo charcoal, and pine charcoal (n=2 for each amount of charcoal and type of charcoal used)

	Log_{10} (colony forming units)		
Charcoal Amount (mg)	Bamboo charcoal	Activated bamboo charcoal	Pine charcoal
2	2.20 ±0.05[a]	2.44 ±0.04[a]	2.40 (±0.01)
6	2.27 ±0.04[a]	2.09 ±0.01[a]	2.28 (±0.13)
10	1.95 ±0.06[b, B]	1.38 ±0.25[b, A]	2.32 (±0.04)[C]
20	ND[c, A]	1.96 ±0.06[c, B]	2.40 (±0.07)[C]

[a–c]Different superscripted lowercase letters indicate differences between mean values in columns (comparison among the amount of charcoal used with each type of charcoal) $P < 0.05$.

[A–C]Different superscripted capital letters indicate differences between mean values in rows (comparison among the types of charcoal used within the amount of charcoal in each sample) $P < 0.05$.

[1]Standard deviation.

ND: no recovered detection of *Salmonella* Typhimurium

Table 8. Body weight, weight gain, and feed to gain ratio of broilers weighed at 14, 28 and 42 d that were fed an addition of bamboo charcoal, activated bamboo charcoal or pine charcoal compared to a basal control diet

	Body Weight (kg)			Weight Gain (kg)				Feed to Gain			
	14 d	28 d	42 d	0 to 14 d	14 to 28 d	28 to 42 d	0 to 42 d	0 to 14 d	14 to 28 d	28 to 42 d	0 to 42 d
Control	0.43 ±0.00[1]	1.62 ±0.01	3.12 ±0.04	0.39 ±0.00	1.20 ±0.01	1.50 ±0.03	3.07 ±0.04	1.24 ±0.01	1.35 ±0.01	1.77 ±0.01	1.54 ±0.00
0.3% Bamboo charcoal	0.42 ±0.00	1.61 ±0.01	3.12 ±0.04	0.38 ±0.00	1.19 ±0.01	1.52 ±0.03	3.07 ±0.04	1.25 ±0.01	1.36 ±0.01	1.76 ±0.02	1.54 ±0.01
0.3% Activated bamboo charcoal	0.43 ±0.00	1.62 ±0.01	3.14 ±0.04	0.38 ±0.00	1.20 ±0.01	1.53 ±0.04	3.09 ±0.04	1.24 ±0.01	1.35 ±0.01	1.77 ±0.02	1.54 ±0.01
0.3% Pine charcoal	0.42 ±0.00	1.59 ±0.01	3.08 ±0.03	0.38 ±0.00	1.18 ±0.01	1.47 ±0.02	3.04 ±0.03	1.25 ±0.01	1.36 ±0.01	1.78 ±0.01	1.55 ±0.01

[1]The values are means ± SEM, n = 12 replicate floor pens containing 21 chicks/pen beginning at placement.

Table 9. Body weight (BW) of Experiment 2 *Salmonella* challenged broilers fed a basal control diet with or without an addition of either bamboo charcoal, activated bamboo charcoal, or pine charcoal from 0 to 42 d[1]

Dietary treatments	BW at 14 d	BW at 28 d	BW at 42 d
Basal Control	0.419 ± 0.003	1.562 ±0.011	3.061 ±0.039
0.3% Bamboo charcoal	0.422 ±0.003	1.563 ±0.011	3.036 ±0.031
0.3% Activated bamboo charcoal	0.429 ±0.004*	1.593 ±0.009*	3.115 ±0.028
0.3% Pine charcoal	0.424 ±0.004	1.570 ±0.005	3.054 ±0.031

[1]The values are means kg ±SEM, n = 10 replicate pens.

[2]At this age only 11-13 birds remained in each pen as the rest had been culled for ceca collection.

*Significantly different ($P < 0.05$) from the negative control within the weigh day.

APPENDICES

Table 10. Composition of the basal control diet for experiment 1, 2, and performance study

Ingredient	Diets		
	Starter (%)	Grower (%)	Finisher (%)
Corn	48.075	52.088	62.035
Soybean meal	43.755	39.594	30.115
Soybean oil	3.796	4.125	3.823
Limestone	1.299	1.269	1.231
Dicalcium Phosphate	1.163	1.065	1.013
Salt	0.293	0.278	0.253
Sodium Carbonate	0.229	0.228	0.225
L-Lys, HCl 78.8%	0.05	0.046	0.069
DL- Met 99%	0.346	0.314	0.24
L-Thr, 98%	0.051	0.052	0.054
Choline Chloride 60%	0.02	0.02	0.02
Quantum Phytase XT 2,500	0.02	0.02	0.02
Vitamin mix[2]	0.227	0.227	0.227
Mineral mix[3]	0.075	0.075	0.075
SolkaFloc[4]	0.6	0.6	0.6
Calculated analysis			
AME (KCal/Kg)	3031	3085	3152
Crude Protein (%)	24.021	22.354	18.643
Calcium (%)	0.95	0.9	0.85
Available Phosphate (%)	0.475	0.45	0.425
Dig Total Sulfur (%)	0.95	0.885	0.741
Dig Lys (%)	1.25	1.15	0.95
Dig Thr (%)	0.812	0.759	0.636

[1]Starter diet fed from day 1 to 14, grower diet from 14 to 28 d,
and the finisher diet from 28 to 42 d.

[2]Vitamin mix provided the following per 100 g of diet: vitamin A, 551 IU; vitamin D_3, 110 IU; vitamin E, 1.1 IU; vitamin B_{12}, 0.001mg; riboflavin, 0.44 mg; niacin, 4.41 mg; d-pantothenic acid, 1.12 mg; choline, 19.13 mg; menadione sodium bisulfate, 0.33 mg; folic acid, 0.55 mg; pyridoxine HCl, 0.47 mg; thiamin, 0.22 mg; d-biotin, 0.011 mg; and ethoxyquin, 12.5 mg.

[3]Mineral mix provided the following in mg per 100 g of diet: Mn, 6.0; Zn, 5.0; Fe, 3.0; Cu, 0.5; I, 0.15; and Se, 0.05.

[4]SolkaFloc was used as an inert filler and additions of charcoal were at its expense.

APPENDIX Table11. Lighting Program for Experiment 1, 2 and the Performance Study

Age (d)	Light Intensity (lux)	Light (h)
0 to 3	20	24
4 to 7	20	20
8 to14	10	16
15 to 28	2	16
28 to 35	2	16
35 to 42	2	23

APPENDIX Table 12: Temperature Protocol for Experiment 1 and 2 and Performance Study

Experimental Day	Temperature (°F) ((°C))
0	93.0 (33.89)
1	92.0 (33.33)
2	92.0 (33.33)
3	90.0 (32.22)
4	90.0 (32.22)
5	89.0 (31.67)
6	88.0 (31.11)
7	88.0 (31.11)
8	87.0 (30.56)
9	87.0 (30.56)
10	86.0 (30.00)
11	86.0 (30.00)
12	85.0 (29.44)
13	85.0 (29.44)
14	84.0 (28.89)
15	84.0 (28.89)
16	83.0 (28.33)
17	82.0 (27.78)
18	82.0 (27.78)
19	81.0 (27.22)
20	81.0 (27.22)
21	80.0 (26.67)
22	80.0 (26.67)
23	79.0 (26.11)
24	79.0 (26.11)
25	78.0 (25.56)
26	78.0 (25.56)
27	77.0 (25.00)
28	77.0 (25.00)
29	76.0 (24.44)
30-42	75.0 (23.89)

APPENDIX Table 13: Pen Randomization 1

Experiment 1 *Salmonella* challenge room

Hall Door

Empty	Empty		Empty	Empty
Empty	Empty		Empty	Empty
Empty	Empty		Empty	Empty
Empty	Empty		Empty	Empty
Pen 1 TRT 1	Pen 9 TRT 2		Pen 17 TRT 4	Pen 25 TRT 1
TRT 4	TRT 1		TRT 3	TRT 2
TRT 2	TRT 3		TRT 1	TRT 4
TRT 3	TRT 4		TRT 2	TRT 3
TRT 1	TRT 4		TRT 2	TRT 1
TRT 2	TRT 1		TRT 3	TRT 4
TRT 4	TRT 3		TRT 1	TRT 2
Pen 8 TRT 3	Pen 16 TRT 2		Pen 24 TRT 4	Pen 32 TRT 3

Outside Door

8 replicate pens/treatment

APPENDIX Table 14: Pen Randomization 2

Experiment 2 *Salmonella* challenge room

Hall Door

Empty	Empty		Empty	Empty
Empty	Empty		Empty	Empty
Pen 31 TRT 4	Pen 21 TRT 2		Pen 11 TRT 3	Pen 1 TRT 1
TRT 3	TRT 1		TRT 4	TRT 2
TRT 2	TRT 4		TRT 1	TRT 3
TRT 1	TRT 3		TRT 2	TRT 4
TRT 4	TRT 2		TRT 3	TRT 1
TRT 3	TRT 1		TRT 4	TRT 2
TRT 2	TRT 4		TRT 1	TRT 3
TRT 1	TRT 3		TRT 2	TRT 4
TRT 4	TRT 2		TRT 3	TRT 1
Pen 40 TRT 3	Pen 30 TRT 1		Pen 20 TRT 4	Pen 10 TRT 2

Outside Door

10 replicate pens/treatment

APPENDIX Table 15: Pen Randomization 3

Experiment 2 Performance room

Hall Door

Pen 49 TRT 1	Pen 61 TRT 2		Pen 73 TRT 3	Pen 85 TRT 3
TRT 2	TRT 3		TRT 4	TRT 4
TRT 3	TRT 4		TRT 1	TRT 2
TRT 1	TRT 1		TRT 2	TRT 3
TRT 1	TRT 2		TRT 3	TRT 4
TRT 2	TRT 3		TRT 4	TRT 1
TRT 3	TRT 4		TRT 1	TRT 2
TRT 4	TRT 1		TRT 2	TRT 3
TRT 1	TRT 2		TRT 3	TRT 4
TRT 2	TRT 3		TRT 4	TRT 1
TRT 3	TRT 4		TRT 1	TRT 2
Pen 60 TRT 4	Pen 72 TRT 1		Pen 84 TRT 2	Pen 96 TRT 3

Outside Door

12 replicate pens/treatment.