ABSTRACT

SANTOS, FERNANDA BOTARO DE OLIVEIRA**.** Impact of poultry age, season, litter quality, and nutritional intervention strategies on *Salmonella* prevalence and populations, serotypes, genotypes, and antibiotic resistance profiles. (Under the direction of Drs. Brian Sheldon and Peter R. Ferket).

Poultry-related salmonellosis is an on-going problem that the poultry industry must continue to address. To address these challenges, *Salmonella* populations present on litter and fecal samples of brooder and grow-out turkey farms were assessed using a quantitative procedure. Furthermore, serotyping, genotyping by pulsed-field gel electrophoresis and antibiotic resistance-susceptibility analyses were used to investigate the diversity of the *Salmonella* serotypes present on these farms. Additionally, alternative on-farm pathogen intervention strategies including feeding whole or coarsely ground grains, increasing insoluble fiber content of the diet and use of an alternative non-litter cage-based housing design (Broilermatic System) were evaluated. *Salmonella* litter populations averaged 2 logs higher in 3-wk turkey samples compared to samples from 19-wk birds. Turkey age also influenced *Salmonella* serotypes, genotypes and antibiotic resistance profiles. Only serovars Javiana and Mbandaka were common between 3 and 19-wk old turkeys. A higher frequency of multidrug resistance was observed in *Salmonella* isolates recovered from samples of 3-wk birds, on average isolates were resistant to >4 antibiotics tested. Supplementing coarse ground corn and increased insoluble fiber (wood fiber) content into the turkey diet did not adversely impact body weights. However, the treatments did not influence *Salmonella* colonization or fecal shedding of turkeys. To examine the impact of housing design and addition of whole or coarsely ground grains on performance, intestinal microbial diversity and *Salmonella* colonization, broilers were reared on four diets consisting of finely or coarsely ground corn and finely ground or whole triticale to market age and *Salmonella* populations measured. Whole grain supplementation decreased *Salmonella* cecal populations while rearing broilers on litter floor as opposed to the Broilermatic System resulted in significant reductions in *Salmonella* cecal populations. Moreover, feeding whole triticale

presumably encouraged the proliferation of bacterial populations which may have competitively excluded *Salmonella* in the ceca of broilers. In conclusion, highly variable *Salmonella* populations and serotypes were detected across all commercial turkey farms and the use of alternative feed ingredients such as triticale may help to reduce *Salmonella* colonization in poultry. Moreover, diet composition and grain coarseness as well as housing design can influence the diversity of the commensal intestinal microflora which may help in the control of *Salmonella* colonization in broiler intestines.

IMPACT OF POULTRY AGE, SEASON, LITTER QUALITY, AND NUTRITIONAL INTERVENTION STRATEGIES ON *SALMONELLA* PREVALENCE AND POPULATIONS, SEROTYPES, GENOTYPES, AND ANTIBIOTIC RESISTANCE PROFILES

by

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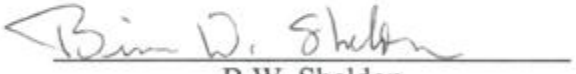
A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

POULTRY SCIENCE
ANIMAL SCIENCE

Raleigh, NC

2006

APPROVED BY:



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DEDICATION

To God and my family

BIOGRAPHY

Fernanda Botaro de Oliveira Santos was born on February 18, 1978 in Araguari, MG, Brazil, where she completed her elementary, secondary, and high school education. In 1995, Fernanda began her undergraduate studies at the Federal University of Uberlândia (*Universidade Federal de Uberlândia*), Uberlândia, MG, Brazil with a major in Veterinary Medicine. During her years in college, Fernanda had many professional experiences including working as an animal pathologist and microbiologist assistant, veterinary consultant, poultry farm and commercial hatchery manager assistant, teaching assistant and veterinary assistant in veterinary hospitals.

After receiving her Bachelor of Science degree in December 1999, Fernanda began working in January of 2002 on the requirements for a Doctor of Philosophy degree in Poultry and Animal Sciences and minors in Food Safety and Biotechnology under the direction of Dr. B. W. Sheldon and Dr. P. R. Ferket. In 2005, Fernanda received an award in recognition of the most outstanding graduate student research paper and presentation at the annual meeting of the Southern Poultry Science Society for her work examining the influence of grain particle size and insoluble fiber content on *Salmonella* colonization and shedding in turkeys fed corn-soybean meal diets. Additionally, she was the recipient of a Certificate of Excellence for her research paper "Influence of housing system, grain type and particle size on *Salmonella* colonization and shedding in broilers fed triticale- and corn-soybean meal diets" at the 2006 Poultry Science Association Meeting.

Fernanda is married to Dr. Anael Santos and God has blessed them with two angels, Amanda and Lucas Santos.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my advisor Dr. Brian W. Sheldon and co-advisor Dr. Peter R. Ferket for their understanding, guidance and encouragement throughout a very challenging and rewarding time of my life. Appreciation is also extended to the other members of my graduate committee, Dr. Donna K. Carver and Dr. Lee-Ann Jaykus for their scientific collaboration, advice, counsel and friendship throughout my doctoral studies and professional development.

I am thankful to Mrs. Annette Israel, Ms. Susan Hale and the staff of the Poultry Science Department of North Carolina State University for their advice, friendship and excellent assistance. Heartfelt thanks are also extended to my fellow graduate students for their time, help and patience.

I would like to thank my parents Eurípedes and Délia Oliveira, my sister Renata, my brother Andre Luíz, and my grandparents Hélia and Delhi Carvalho for the love and help that only family can provide. Also, I would like to thank my parents-in-law, Mr. Anael Santos and Mrs. Maria Bernadete Santos, for their love and support during this study.

Most importantly, I wish to express thankfulness, love, and gratitude to my husband Anael for his love, faith, encouragement, affection, happiness, and understanding; and to my two special angels, Amanda and Lucas for their unconditional love.

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LITERATURE REVIEW

1.1 INTRODUCTION

Achieving the genetic potential for growth, maintaining health and food safety at the lowest input costs are the goals of commercial poultry producers throughout the world. The poultry industry has undergone remarkable changes and growth over the last 30 years and it will likely continue to grow in the next decade. Production of poultry meat is expanding to meet higher demand for low-cost, healthy and convenient products. Poultry production is one of the most efficient industries in the modern world; however, poultry contamination with foodborne pathogens, poultry-related foodborne illness and subsequent consumer productivity losses are on-going challenges that the poultry industry faces. The changes that have occurred in the food industry over the last two decades, such as centralized production and large-scale distribution, has been accompanied by a doubling in human salmonellosis incidence (Altekruse et al., 1997). The USDA Economic Research Service (ERS) has estimated the annual costs due to foodborne *Salmonella* infections at 2.4 billion dollars (USDA, 2004). A significant number of these human infections have been associated with the consumption of contaminated poultry products (Daniels et al., 2002; Thorns, 2002). A number of investigators have reported a high incidence of *Salmonella* contamination on retail chicken and turkey carcasses (Zhao et al., 2001; Logue et al., 2003; Fratamico, 2003).

The United States population is a large consumer of poultry meat. The Foreign Agricultural Services has estimated that U.S. poultry consumption increased 17.4% since 2000 (USDA, 2006a). Broiler meat consumption alone has increased approximately 30% in the last six years, and it is expected that Americans will consume about 13,878 tons of chicken meat in 2006 (USDA, 2006a). This same report shows that turkey meat consumption has been more consistent and is projected for 2006 to be approximately 2,208 tons in the

United States. As consumers have become more concerned about foodborne disease and perceived risks of foodborne infections have increased, control and possible elimination of *Salmonella* has become an important goal of the poultry industry (Guo et al., 1999), especially at the pre-harvest level. Prevention and reduction of *Salmonella* contamination should start at the farm, which ultimately should minimize pathogen contamination at the processing plant. However, before HACCP-type food safety programs are extended to the production phase, considerable research must first be conducted to assess the current populations and prevalence of pathogens present on the farm and to identify critical control points and intervention strategies that effectively reduce or eliminate *Salmonella*. While numerous studies have determined the prevalence of *Salmonella* contamination during the grow-out phase (Read et al., 1994; Nayak et al., 2003), very few studies have actually measured *Salmonella* populations because of time and cost considerations.

In order to prevent or eradicate *Salmonella* from poultry farm environments, it is necessary to prevent its introduction or re-introduction onto the farm. To accomplish this goal it will be necessary to identify all possible transmission routes and to determine how this organism is able to persist on the farm. Because carcass contamination with *Salmonella* has been linked to colonization of live birds on the farm, further reductions in product contamination at the processing plant level will require effective on-farm interventions. Several laboratory methods have been used to better understand the behavior and transmission of salmonellae at the pre- and post-harvest stages including standard culture methods, molecular biology and serological typing. Many of the standard culture methods employed include a preliminary enrichment step since low *Salmonella* populations are generally present in environmental and food samples (Nivas et al., 1973; Cox et al., 2000,

Bailey et al., 2001). An alternative culture method that can be used to estimate bacterial populations is the most probable number (MPN) procedure. Although more costly and laborious, the MPN procedure is very sensitive (detection limit of 10 bacterial cells/gram of sample) and can provide good quantitative data from environmental samples (Santos et al., 2005). Bacterial typing methods such as serotyping, pulsed-field gel electrophoresis (PFGE), antibiotic resistance patterns (AbR), plasmid profiling, and other procedures have been used in epidemiological investigations to elucidate the origin and mode of transmission of pathogens (e.g. *Salmonella* outbreaks). Once the route of transmission has been identified, control measures can be implemented to avoid or reduce contamination or prevent recontamination. Therefore, studying the phenotypic and genotypic characteristics of *Salmonella* isolates can help to understand the transmission pathways of this pathogen throughout all sectors of the poultry industry.

Despite food safety issues, the modern commercial broiler and turkey continues to show increased yearly genetic gains. This selection for increased growth rate has resulted in changes in the gastrointestinal development of these animals (Tottori et al., 1997). Commercial poultry species are susceptible to a number of enteric health problems which can have a profound impact on growth performance of poults and chicks as it affects feed digestion, nutrient absorption, and mortality (Tottori et al., 1997). In addition, poor intestinal health is associated with an increased susceptibility to infectious diseases and colonization by enteric foodborne pathogens (Patterson and Burkholder, 2003). Therefore, compromised intestinal health adversely impacts the poultry industry by reducing animal productivity and welfare and by increasing the potential for contamination of poultry products marketed for human consumption.

An important means of preventing human salmonellosis is by preventing infection of poultry (Porter, 1998). Intestinal competitive exclusion products, probiotics, prebiotics, organic acids and enzymes have all been used to reduce or eliminate *Salmonella* and other pathogens in live poultry. Interestingly, the use of alternative grains or cereals has been shown to influence the ecology of the intestinal tract of poultry, including *Salmonella* colonization. Bjerrum and coworkers (2005) have previously demonstrated that inclusion of whole wheat in the diet of broilers decreased intestinal *Salmonella* colonization. Similar population reductions were also reported when turkeys were fed wheat- or triticale-based diets (Santos, 2006). Altering feed structure by changing grain particle size is another approach for controlling salmonellae in the poultry industry (Bjerrum et al., 2005; Huang et al., 2006). Improvements in feed efficiency (Plavnik et al., 2002; Lentle et al. 2006) and nutritive value (Svihus et al., 2004) has been reported when broilers were fed whole wheat-based diets. Besides affecting performance and nutrient uptake, changing feed structure was reported to influence *Salmonella* infection of broilers in some studies. Bjerrum et al. (2005) showed that broilers fed pelleted feed had a higher *Salmonella* population in the gizzard compared to those that were supplemented with whole wheat. Similar results were reported by Huang and coworkers (2006) who reported an increased incidence of *Salmonella* Typhimurium in the gizzard and cecal contents of broilers fed pelleted feed. These results suggested that feed structure may influence *Salmonella* colonization by changing the gastrointestinal ecology of broilers.

Another subject of considerable concern for the poultry industry is the generation of large volumes of waste in the form of poultry litter as well as the sustaining of infections through recycling of pathogens due to direct contact with contaminated litter. One recently

developed alternative for pathogen control, promoting bird growth and reducing wastes is the use of non-litter housing systems. The Farmer Automatic Broilermatic Cage System is an example of a non-litter hosing system. The system is a cage facility that allows broilers to be raised on a surface nearly free of feces thus reducing the potential for recontamination with pathogens shed in their waste. Broilers raised in this system have demonstrated increased weight gains, decreased mortality rates, and a reduction in the overall volume of waste produced since no manure carrier (i.e., wood shavings) is required (Havenstein et al., 1998).

The avian intestinal tract is complex and its normal function is dependent on the interaction between the bird and its intestinal microflora, which is a relatively under-explored ecosystem. A better understanding of the intestinal ecosystem could result in a greater opportunity to prevent colonization of unfavorable microbes and reduce the incidence of a variety of gastrointestinal tract (GIT) diseases (Rastall et al. 2005). The intestinal microflora is composed of a very diverse collection of culturable and non-culturable microbial species. Much of the knowledge on intestinal bacterial species has been determined using standard culture methods. Although culture-based techniques have been useful for analysis of specific groups of bacteria, it has several limitations for surveying the intestinal ecosystem such as time- and labor-intensive constraints, and the use of selective culture media that dictates the types of bacteria that can be recovered (MacCracken et al., 2001).

The use of molecular biology methods has greatly enhanced the knowledge of gastrointestinal bacterial communities. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons is a quick, economical, and reliable technique for the analysis of microbial community fingerprint (Muyzer et al., 1993). In addition, this cultivation-independent technique is less labor-intensive than traditional microbiological approaches and

can be applied to evaluate dietary-, drug-, or disease-associated alterations of intestinal microbial populations (McCracken et al., 2001). The DGGE method is based on the analytical separation of DNA fragments of identical or near-identical lengths but with varying sequence compositions. Changes in fragment mobility are associated with partial melting or the denaturing of DNA sequences in discrete regions, the so-called melting domains. When the DNA enters a region of the gel containing sufficient denaturant, a transition of helical to partially melted molecules occurs, and migration is severely retarded. Sequence variation within such domains alters the melting behavior, and sequence variants of the different amplification products stop migrating at different positions in the denaturing gradient (Liu and Stahl, 2002). Furthermore, DNA can be collected from bands on the DGGE gel which can be subsequently sequenced for phylogenetic analysis (Høj et al., 2005).

In conclusion, a better understanding of the behavior and ecology of *Salmonella* and of the avian intestinal ecosystem will result in a greater opportunity to develop intervention strategies to control *Salmonella* in the poultry production environment as well as to reduce the incidence of a variety of GIT infections of poultry. Considering that some feed ingredients and the use of coarser diets can potentially regulate the avian intestinal environment towards a healthier state, it should be possible to manipulate the intestinal microbial community by the use of coarsely ground feed ingredients such as cereal grains that are rich in non-digestible but fermentable carbohydrates.

The following review of the scientific literature was focused on aspects of *Salmonella* epidemiology, survival and transmission, as well as on characteristics that might influence pathogen colonization of the poultry GIT and the occurrence of human salmonellosis. Concepts required for enhancing avian intestinal health with an emphasis on nutritional

strategies to modulate microbial ecology and to discourage *Salmonella* intestinal colonization were addressed. Although it was not the purpose of this dissertation to review the extensive literature on salmonellosis and its consequences on intestinal health of poultry and humans, a general review is necessary to further comprehend the connection between avian colonization and human disease, and how the use of high-fiber cereals and the level of coarseness of the diet may affect the intestinal microbial community of poultry.

1.2 THE GASTROINTESTINAL TRACT OF POULTRY

Digestion is a complex process involving several different chemical and physical processes whose major function is to transform feed such that nutrients are released and subsequently absorbed supplying the animal's requirements for maintenance, growth, and reproduction. Feed is ingested, broken down into smaller particles, macerated, mixed with digestive enzymes, and propelled through the digestive tract by the muscular activities of the tract. Salivary, gastric, pancreatic, biliary, and intestinal secretions collectively provide mucus for protection and lubrication of the tract, enzymes that aid in digestion, a watery medium, and optimal pH required for digestion. Microorganisms, indigenous to the digestive tract, can provide additional nutrients by breaking down unconsumed and indigestible compounds and by synthesizing amino acids and vitamins essential to the host animal.

In the avian species, food is stored in the crop and is gradually delivered to the proventriculus and ventriculus (gizzard). Among the few types of bacteria that rapidly colonize the crop, *Lactobacillus*, predominantly *Lactobacillus salivarius* (Sarra et al., 1985), coliform bacteria, *Streptococcus* and *Enterococcus* are some examples (Fuller, 1977). The pH of the crop is generally more acidic (ca. 5.0) due to lactic acid production mainly by

Lactobacillus, which may also exert bacteriostatic and bactericidal properties (Fuller, 1977) that appear to reduce the colonization of this organ by bacteria that are not very acid tolerant such as *Escherichia coli* and *Salmonella* (Fuller, 1977). Some bacterial degradation of starch occurs in the chicken crop, however, any significant fiber digestion may be limited by the low pH.

Chemical and physical digestion of food occurs primarily in the proventriculus and gizzard, respectively (Duke, 1986). The major function of the proventriculus is to chemically digest the food through acid production, mainly hydrochloric acid; whereas the gizzard serve as the grinding organ. In the gizzard, where rhythmic contractions of the walls macerate and move the feed, feed is ground to a fine particle size (100 μm or smaller) and conveyed along the gizzard crevices through the pylorus sphincter to the duodenum (Hetland et al., 2002). To increase the efficiency of peptic digestion, the gastric reflux mechanism moves digesta from the gizzard back into the proventriculus for each contraction. Both the proventriculus and gizzard environment appear to be unfavorable for microbial growth considering that the pH ranges from 1 to 4, thus any surviving microorganisms must show a degree of acid tolerance. Despite this unfavorable environment, bacterial populations have been observed in the gizzard. In a study of the chicken GIT, Smith (1965) found *Lactobacillus* at 10^8 colony forming units per gram (cfu/g) of gizzard contents, with much lower numbers of *E. coli*, *Streptococcus*, *Enterococcus* and yeasts.

The small intestine, composed of duodenum, jejunum and ileum, is responsible for most of the digestion and absorption of nutrients (Turk, 1982). The avian intestinal tract is a multilayered tube composed of a serosal layer, a longitudinal and a circular muscular layer, and a submucosal and mucosal layer (Turk, 1982). Feed is digested in the lumen of the small

intestine by the pancreatic, biliary and intestinal wall enzymes and followed by nutrient absorption which takes place primarily through the mucosa (Turk, 1982). The interior surface of the intestine is folded into numerous complex structures called villi (Romanoff, 1960) and between the villi are the crypts of Lieberkuhn where crypt cells proliferate and then migrate up the villi (Turk, 1982). These cells have a life cycle of 48 to 96 hours under normal conditions (Turk, 1982; Moran, 1985; Uni et al., 1998). As the crypt cells move up the villus they differentiate into principal (absorptive) or goblet (secretory) cells (Turk, 1982). The goblet cells produce mucopolysaccharides that are secreted into the intestinal lumen producing a layer that covers the villar surface (Moran, 1985). This mucus layer serves as a protective barrier for the delicate absorptive surface of the intestine. The major component of the mucous layer is the mucin which serves as a lubricant of the digesta (Moran, 1985). Mucin also discourages the translocation of harmful microorganisms, binding of chemical irritants, and provides a medium for the colonization of favorable microflora (Thompson and Applegate, 2005). Moreover, the mucin layer also accumulates bactericidal and bacteriostatic compounds capable of neutralizing or killing bacteria. As the loosely adherent mucus layer is sloughed off it traps and removes resident or invading bacteria from the intestinal tract (Thompson and Applegate, 2005). Therefore, it is generally accepted that the mucin layer is an important component of a healthy intestinal tract which helps to maintain a strong barrier against pathogen invasion.

The ceca originate at the junction of the small and large intestine and vary considerably in size and form between bird species (Józefiak et al., 2004). The flow of material into the ceca is controlled by valves which usually only allow fluids and fine particles to enter. In wild galliforms, the ceca are evacuated only about once per 24 hours,

thus, the appendages provide relatively stable conditions for microbial proliferation (Duke, 1986). The resultant microbiota tends to be large and diverse, reaching a population of around 10^{11} cfu/g of cecal content. The ceca are considered the principal fermentation chambers in the avian GIT where they play an important role in water absorption, the microbial degradation of some carbohydrates, microbial synthesis of vitamins and degradation of nitrogenous compounds (Józefiak et al., 2004). The size of the ceca is directly related to the degree of fermentation that occurs in the organ, such that the most developed avian ceca are found in granivores and species of birds whose diets contain high levels of plant fiber or chitin (Stevens and Hume, 1998).

The intestinal microflora of poultry has been extensively investigated using culture-based methods (Salanitro et al., 1974; Mead and Adams, 1975; Salanitro et al., 1978). These studies documented that the predominant flora of the ileum included *Lactobacillus*, *Streptococcus*, *E. coli*, *Enterococcus*, and *Staphylococcus* at about 10^9 cfu/g of ingesta whereas in the ceca the populations reached up to 10^{11} cfu/g of cecal content with the dominant microorganisms being *Eubacterium* and *Bacteroides*. Other obligate anaerobes were also present, especially in the ceca, including anaerobic cocci and species of *Propionibacterium, Clostridium, Gemmiger* and *Fusobacterium*. The ceca contain the largest number of bacteria in the chicken GIT with several of these genera being strict anaerobes (Barnes, 1979). Although over 200 different bacteria have been isolated from cecal contents, it has been reported that only 10-60% of the bacteria in the ceca can be cultivated using anaerobic culture techniques (Zhu et al., 2002).

New molecular analyses have expanded the knowledge base on ileal and cecal bacterial populations. Using molecular analysis of 16S ribosomal DNA (rDNA) amplicons

from combined ileal bacterial samples (digesta and mucosal), Gong et al. (2002) reported that the bacterial populations in the ileum were largely gram-positive including *Lactobacillus* and *E. cecorum*, which represented more than 70% of the ileal mucosal and lumen populations. Forty-nine percent were related to *Lactobacillus aviaries*. The findings from this study also demonstrated that butyrate-producing bacteria (including those related to *Fusobacterium prausnitzii*), ruminococci, clostridia and *E. cecorum* were the predominant groups of bacteria found in the cecal mucosa. Butyrate is a short-chain fatty acid and the principal energy source for the cell lining of the lower intestine where it helps to control the growth and overall metabolic activity of colonic cells (Brouns et al., 2002). Reduced supply of butyrate to colonic cells causes intestinal atrophy and impairs function, including reduced immune response (Scheppach and Weiler, 2004). An additional benefit of butyrate concerns its stimulatory role on commensal intestinal microflora and the competitive exclusion of pathogens (Brouns et al., 2002). Similar results were reported by Lu and coworkers (2003) who detected predominantly lactobacilli in the small intestine, and low numbers of streptococci and enterobacteria; the cecal flora was composed mainly of anaerobes and fewer numbers of facultative bacteria.

1.2.1 Intestinal Microbial Diversity

Intestinal bacteria influence health through their effects on intestinal morphology, nutrition, pathogenesis of intestinal disease, and immune responses (Lu et al., 2003). However, many factors can affect the composition of the avian bacterial community such as diet, feed additives, age, and antibiotic administration (Knarreborg et al., 2002). The animal's intestine is the natural habitat for a large and dynamic bacterial community which reflects the

coevolution of microorganisms with their animal host and the diet adopted by the host. Thus, microbial diversity encompasses the spectrum of variability among all types of microorganisms in the natural world and as altered by any kind of intervention. Microbial diversity can be studied in different environments, including in the intestinal tract of animals, by investigating the association between the many different microorganisms of the intestinal flora and the host.

1.2.2 Methods of Studying Intestinal Microbial Diversity

In the late 19th century the practical success of culture methods for the identification of bacteria led to the general belief among medical scientists that all bacteria are cultivatable by existing methods. Microscopic counts of native samples and subsequent colony counts after cultivation may however differ by more than 2 $\log_{10}$ units (Simon et al., 2004). This observation shows that cultivation methods have not yet reached their full potential and may never be able to allow growth of all bacteria within a given sample. Consequently, insight into the interaction between the host and the microbial community and how environmental factors influence the microflora has been limited by the exclusive use of cultural procedures for the study of microbial diversity (Zoetendal et al., 1998).

The use of molecular biology methods has greatly enhanced the knowledge of gastrointestinal bacterial communities. One major advantage of molecular techniques is the rapidity and sensitivity of the determination as compared to culture methods. Ribosomal DNA (rDNA) and RNA (rRNA) have been shown to be excellent markers in order to group bacteria according to their phylogenetic origin (Lane et al., 1985). Comparison of bacterial rDNA sequences has demonstrated similarities that can be categorized into phylogenetic

trees which correlate well with existing taxonomic systems and emphasize relationships that have led to the generation of new taxa (Simon et al., 2004). Currently, ribosomal RNA (rRNA) or DNA (rDNA) analysis is the most commonly used measure of environmental diversity (Gong et al., 2002; Zhu et al., 2002; Lu et al., 2003). Species diversity consists of two components, species richness and species evenness (distribution). Species richness (or species abundance) is used to describe the number of species present, whereas species evenness (or species equitability) is used to describe how evenly individuals are distributed among these species (Hill, 1973). Certain attributes of the rRNAs favor their use as molecular markers. The genetic sequences that code for rRNA are among the most highly conserved (Woese, 1987). The rRNA can be viewed as composed of structural domains in which sequence variation differs with respect to increasing phylogenetic distance. The accuracy of phylogenetic inference is dependent not only upon the number of bases compared, but also upon the particular regions of the molecule compared. The rRNAs of many eukaryotes and some prokaryotes differ significantly in size. The small-subunit rRNA and large-subunit rRNA of bacteria are referred to as 16S and 23S rRNAs, respectively. The small subunit rRNA (ca. 1,500 nucleotides) provides a large amount of information useful for phylogenetic inference, thus it has become the established reference for phylogenetic inference because it is much easier to sequence (Liu and Stahl, 2002).

Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons is a suitable technique for the analysis of microbial community fingerprints (Muyzer et al.,1993). The DGGE method is based upon the analytical separation of DNA fragments of identical or near-identical lengths but with varying sequence compositions. Separation is based on the changing in electrophoretic mobilities of DNA fragments migrating in a gel containing a

linearly increasing gradient of DNA denaturants, usually urea and formamide. Changes in fragment mobility are associated with the denatured DNA sequences in discrete regions, the so-called melting domains. When the DNA enters a region of the gel containing sufficient denaturant, a transition of helical to partially denatured molecules occurs and migration is severely retarded. Sequence variation within such domains alters the denaturing behavior and sequence variants of the different amplification products stop migrating at different positions in the denaturing gradient (Liu and Stahl, 2002). DNA can be collected from bands on the DGGE gel which can be subsequently sequenced for phylogenetic analysis (Høj et al., 2005). This technique has been commonly used to profile the intestinal microbial community of humans (Liu and Stahl, 2002), mice (MacCracken et al., 2001), broiler chickens (Gong et al. 2002; Lu et al., 2003), composting processes (Ishii and Takii, 2003) and soil (Kirk et al., 2004). However, the DGGE technique has been shown to select only 90 to 99% of the total microbial population because less dominant bacteria do not form visible DGGE bands which could then be selected using other molecular approaches, such as cloning (Zoetendal et al., 1998; Konstantinov et al., 2003). While molecular methods are valuable tools for investigating the diversity and structure of bacterial communities, combining different methods that complement each other is a more useful strategy for monitoring changes in microbial communities and ecosystems (Torsvik et al., 1998).

1.3 *SALMONELLA* AND FOODBORNE SALMONELLOSIS

1.3.1 History

Salmonella was first isolated in the United States in 1885 by Salmon and Smith from swine suffering from hog cholera and was named *Bacillus cholerae-suis* (Le Minor, 1981).

However, long before its first isolation *Salmonella* was the cause of serious disease. In the early 19th century the connection between human intestinal ulceration and a contagious agent, later identified as typhoid fever, was first documented by French pathologists (D'Aoust, 2001). Later European studies led to the isolation and characterization of the typhoid bacillus and the development of a serological diagnostic test to detect this pathogenic agent (Le Minor, 1981). This information was subsequently used to identify closely related organisms identified as paratyphoid agents. The generic term *Salmonella* was first used by Lignières in 1900 after the development of improved serological techniques that enabled the identification of somatic (O) and flagellar (H) antigens (Le Minor, 1981). In 1926, the first antigenic scheme for the classification of salmonellae was proposed by White, which was expanded by Kauffmann in 1941 to subsequently become the Kauffmann-White Scheme (D'Aoust, 2001).

National surveillance for *Salmonella* infections was established in 1962 following recognition of the importance of this pathogen as the cause of potentially preventable infectious disease in the United States (Chalker and Blaser, 1988). Recently, nontyphoidal salmonellosis has been one of the most commonly reported bacterial infections in the United States (CDC, 2006), with the majority of these cases being of a foodborne origin (Mead et al., 1999). Although substantial progress has been made in foodborne disease prevention, the epidemiology of *Salmonella* has undoubtedly contributed to an increased frequency of outbreaks of infections linked to food.

1.3.2 Taxonomy

The nomenclature of the genus *Salmonella* has been the subject of debate for decades. Currently, the use of two systems of nomenclature for the members of this genus has been

the source of confusion and discrepancies (Tindall et al., 2005). In 1987, Le Minor and Popoff proposed that the type species of the genus *Salmonella* should be changed to *Salmonella enterica*, with the type strain being LT2 (Le Minor, 1987). Finally in 2005, after taking into consideration the requests of Le Minor et al. (1987) and the requests for an opinion by Euzéby (1999) and Ezaki et al. (2000a,b), the Judicial Commission has ruled that *Salmonella enterica* Le Minor and Popoff 1987 becomes the type species for the genus *Salmonella* Lignieres 1900, replacing *Salmonella choleraesuis* (Judicial Commission, 2005). Moreover, to avoid misinterpretation of the Code, the epithet *enterica* in *Salmonella enterica* is conserved over all earlier epithets that may be applied to this species. The Judicial Commission also ruled that the subspecies combinations proposed by these authors should be considered validly published and consequently the *Salmonella enterica* subsp. *enterica* was automatically created (Tindall et al., 2005). Besides considering the request of Le Minor et al. (1987), the Judicial Commission has also taken into consideration the taxonomic proposal of Revees et al. (1989). Following both taxonomic proposals, only two species are to be recognized for the genus *Salmonella*, which are *enterica* and *bongori* (Tindall et al., 2005). Therefore, the taxonomy and resulting nomenclature from the recent ruling of the Judicial Commission (2005) together with both taxonomic interpretations (Le Minor et al.,1987 and Revees et al.,1989) are described on Table 1.

Table 1. *Salmonella* species and subspecies according to Lignieres 1900 taxonomy[1]

Genus	Species	Subspecies
Salmonella	*enterica*	*enterica* *arizonae* *diarizonae* *houtenae* *indica* *salamae*
	bongori	-

[1] Judicial Commission (2005).

Salmonella serotyping is based on the immunoreactivity of two surface antigens, "O" and "H". The O antigen is the outermost polysaccharide component of the lipopolysaccharide-protein chain exposed on the cell surface (CDC, 2005a). The H antigen is a protein antigen known as flagellin; multiple flagellin subunits compose the filament component of the flagella. The flagellin molecule is composed of two regions; a conserved region which gives the flagellin its characteristic filament structure and a variable region which is surface exposed (CDC, 2005a). *Salmonella* is unique among enteric bacteria in that it can express two different flagellin antigens. The two antigens are referred as Phase 1 and Phase 2 and they are usually expressed one at a time in a single bacterial cell (CDC, 2005a). Some *Salmonella* isolates express only one flagellin antigen and they are classified as monophasic. Currently, there are 2,463 *Salmonella* serotypes identified, of which 59% of them belong to *Salmonella enterica* subsp. *enterica*. Ninety-nine percent of human and warm-blooded animal infections are caused by this subspecies (Brenner et al., 2000). The other five *Salmonella enterica* subspecies and *Salmonella bongori* are usually environmental or cold-blooded animal isolates and rarely isolated from humans (Brenner et al., 2000).

There are many benefits of adopting the nomenclature changes recently ruled. The

first is that the approved taxonomy is currently in use by the World Health Organization (WHO) and by the U. S. Centers for Disease Control and Prevention (CDC). Secondly, by having a final judicial decision that is well explained to avoid ambiguities and misinterpretation, the chances of having the new nomenclature widely accepted are greatly improved. For the purpose of this document serotypes of *Salmonella enterica* subsp. *enterica* will be referred as *Salmonella* accompanied by the serotype name. For example, *Salmonella enterica* subsp. *enterica* ser. Typhimurium will be referred as *Salmonella* Typhimurium (or *S.* Typhimurium).

1.3.3 Physiology & Survival

Salmonella species are gram-negative, 2-3 μm long, facultative anaerobic, nonsporulating rod-shaped bacteria. The genus *Salmonella* is known to be comprised of versatile microorganisms that easily adapt to extreme environmental conditions (D'Aoust, 2001). These bacteria have broad physiological limits for growth; for example, their growth temperatures range from 7° to 48°C (optimum 37°C), pH ranges from 4 to 9.5 (optimum 6.5-7.5), and water activity (a_W) above 0.93 (optimum 0.96) (Jay, 1986; D'Aoust, 2001). The wide range of temperature that enables salmonellae to grow raises the concerns about the efficiency of some preventive methods used by the food industry, namely chilling and refrigerated storage (D'Aoust, 2001). Some strains of salmonellae were shown to have psychrotropic characteristics by being able to grow in foods stored at 2 to 4°C (D'Aoust, 1991). Thermotolerance and the synthesis of increased concentrations of heat-shock proteins have also been demonstrated in *Salmonella* Typhimurium (Foster et al., 1995). Moreover, another study indicated that prolonged exposure of mesophilic strains to temperatures above

their optimum for growth resulted in mutants of *S.* Typhimurium capable of growth at 54°C (Droffner and Yamamoto, 1992). Starvation and acid shock can also induce the production of heat-shock proteins by *Salmonella* Typhimurium, however, adaptation to adverse conditions other than these may not produce cross-protection to heat (Foster et al., 1995). This capability of cross-protection is also a concern for the food industry since many food products are preserved by addition of acidifiers followed by a heat treatment.

Acidic pH is one of the most frequent stress conditions encountered by microorganisms (Foster et al., 1995). An acid tolerance response displayed by *Salmonella* species was previously demonstrated by preconditioning wild-type strains on pH gradient plates followed by growth in liquid and solid media at considerably lower pH values than the parent strains (Foster et al., 1995; D'Aoust, 2001). This response was not attributed to a mutation because these cells lost their acid resistance after passage through a neutral pH medium (Foster et al., 1995). Acid tolerant bacteria are a concern for the food and poultry industry because they raise concerns about the safety of fermented foods and raw milk products (D'Aoust, 2001) and about the efficacy of most litter treatments which are primarily based on acidifiers. The acidification of food products or poultry litter could provide a favorable environment for the growth of endogenous salmonellae with increased acid resistance. The outcomes of these scenarios could include contamination of ready-to-eat products and dissemination of acid tolerant *Salmonella* strains across consecutive poultry flocks. However, previous studies have reported that litter *Salmonella* populations decreased when litter pH was more acidic (Payne et al., 2002; Santos et al., 2005). Certainly, the degree of reduction in bacterial populations and the potential to develop tolerance to an acidic environment will depend on other factors such as the exposure time or exposure to other

components in the medium such as fats which may create a protective barrier.

Addition of salt to food products has been used for centuries to prevent food spoilage. The preservative effect of high salt concentrations is a result of a significant decrease in a_W causing bacterial lysis (D'Aoust, 2001). However, it has been shown that bacterial salt tolerance increases when the temperature rises from 10 to 30°C, but the magnitude of this adaptive response is food and strain dependent (D'Aoust, 1989). At the poultry production level, keeping the litter dry is one of the most important management practices. Wet litter can increase the susceptibility to respiratory disease by increasing ammonia levels (Poss, 1998) and wet litter is also highly correlated with the occurrence of foot pad dermatitis (Wang et al., 1998; Mayne et al., 2004). Some field studies have shown that low-a_W litter or chicken manure, specifically lower than 0.84, can significantly reduce or limit *Salmonella* growth (Himathongkham et al., 1999; Hayes et al., 2000). However, Santos and coworkers (2005) have isolated *Salmonella* from litter samples with a_W lower than 0.84. Moreover, the same study showed that the population of isolates present in the low-a_W litter samples ranged from 1.4 to > 4.7 log/g (MPN). Although previous studies have emphasized the increased ability of *Salmonella* to grow under low water activity, acidic conditions and increasing temperature environments (D'Aoust, 1989, Santos et al., 2005), the capacity to adapt to stressful situations will depend on the specific conditions the bacterium is exposed to. For example, the presence of salt in acidified foods can decrease the antibacterial activity of organic acids and anaerobiosis can induce salt tolerance in salmonellae (D'Aoust, 2001). The same principle could be applied to the complex environment of poultry houses where the intrinsic and extrinsic characteristics of the litter and the bird's intestinal tract peculiarities can drastically influence growth kinetics of *Salmonella* species.

1.3.4 Methods of Salmonella Detection

The method of analysis employed can have a considerable effect on isolation of *Salmonella* (Scotter et al., 1993; Uyttendaele and Debevere, 1996). In addition, the likelihood of detecting this pathogen is influenced by the sample and media type and incubation conditions (Jørgensen et al., 2002).

In 2004, the Center for Food Safety and Applied Nutrition (CFSAN) proposed a method to detect *Salmonella* in environmental samples from poultry houses (FDA, 2004). The method is based on pre-enrichment of the sample in buffered peptone water (BPW) followed by incubation at 35°C for 24 $\pm$ 2 h. After incubation, 1 ml and 0.1 ml of the pre-enrichment broth are transferred to 10 ml of tetrathionate (TT) broth and 10 ml of Rappaport-Vassilliadis (RV) medium, respectively. The selective enrichment tubes are then incubated at 42°C for 24 $\pm$ 2 h. Following incubation, one loopful of each selective enrichment broth (TT and RV) is streaked onto brilliant green agar supplemented with novobiocin (BGN), xylose lysine tergitol 4 (XLT4) agar and bismuth sulfite (BS) agar plates. Presumptive positive colonies are stabbed and streaked onto lysine iron agar (LIA) and triple sugar iron (TSI) agar slants. Complementary confirmation testing including serology is described by Andrews and Hammack (1998).

Many of the standard culture methods employed to recover *Salmonella* species include an enrichment step since low *Salmonella* populations are generally present in environmental and food samples (Nivas et al., 1973; Cox et al., 2000; Bailey et al., 2001). Although there is reasonable consensus on the performance of this *Salmonella* isolation technique, available data on the populations of this pathogen at the poultry production level are scarce due to excessive time and cost considerations. An alternative culture method that

can be used to estimate bacterial populations is the most probable number (MPN) procedure. This method is based on 3 different steps that simultaneously inhibit growth of other competitive microflora and allow recovery of injured *Salmonella* cells. The first step, pre-enrichment, recovers injured cells but avoids bacterial growth since the medium has a low nutrient content. The selective enrichment step follows and is based on the addition of chemicals to the medium that will inactivate most background microflora yet preserve the target bacteria. The last step which is the selective and differential plating is based on using selective chemical agents and other components in the media that enable the differentiation of *Salmonella* colonies from other bacterial contaminants. Although more costly and laborious, the MPN procedure is very sensitive (1 log MPN/gram of sample) and can provide good quantitative data from environmental samples (Santos et al., 2005).

Molecular techniques based on amplification of specific DNA sequences by polymerase chain reaction (PCR) have been increasingly used to identify pathogens from poultry-related samples (Soumet et al., 1999; Oliveira et al., 2002). The use of PCR reduces the time of detection and increases sensitivity and specificity of diagnostic methods (Oliveira et al., 2002). *Salmonella* Enteritidis has been identified by PCR in turkey semen using genus species-specific primers (Donoghue et al., 2004). Although there are many advantages of using PCR-based techniques to detect salmonellae from poultry samples, the procedure only confirms the presence or absence of the pathogen and lacks enumeration capabilities.

1.3.5 Methods of Salmonella Characterization

Bacterial typing methods such as serotyping, pulsed-field gel electrophoresis (PFGE), ribotyping, antibiotic resistance patterns (AbR), plasmid profiling, multilocus sequence

typing (MLST) and other procedures have been used in epidemiological investigations to elucidate the origin and mode of transmission of *Salmonella*. Studying the phenotypic and genotypic characteristics of *Salmonella* isolates can help to understand the transmission pathways of this pathogen within the poultry industry and possible epidemiological links with clinical isolates.

Serotyping is an identification tool used by the United States government to study *Salmonella* epidemiology. The Public Health Laboratory Information System (PHLIS) surveillance data has shown that the most common *Salmonella* serotypes isolated from human sources in 2003 were *Salmonella* Typhimurium, Enteritidis and Newport. In comparison, *Salmonella* Senftenberg, Hadar and Heidelberg, and *Salmonella* Heidelberg, Kentucky and Typhimurium were the three most isolated serotypes from turkey and broiler samples, respectively (CDC, 2004). Even though serotyping has been a useful technique to differentiate salmonellae, it is not discriminatory enough to identify the source of a common *Salmonella* serotype, such as *S.* Typhimurium and Enteritidis, within a food production system. Clearly, a single method cannot be relied upon for discriminating between strains and studying the ecology of *Salmonella*.

Pulsed-field gel electrophoresis is a very discriminating and reproducible typing method which is commonly used in epidemiological investigations. In 1996, the Centers for Disease Control and Prevention established the PulseNet which is the national molecular subtyping network for foodborne disease surveillance (Swaminathan et al., 2001). The PFGE process is based on restriction enzymes that cut the chromosomal DNA infrequently and thus generate a small number of large DNA fragments which can be effectively resolved by the PFGE process (CDC, 2005b). Previous studies have shown that PFGE provides valuable

information on differentiating *Salmonella* serotypes and allows detection of DNA polymorphisms that were previously undetected by other techniques such as ribotyping (Liebana et al., 2002; Nayak et al., 2004).

Other molecular techniques that can be used in conjunction with PFGE are the random amplification of polymorphic DNA (RAPD), plasmid profiling and multilocus sequence typing (MLST). The RAPD technique is based on the use of random primers that are able to anneal and prime at multiple locations throughout the genome and produce a spectrum of amplified products which are characteristic of the template DNA. The resultant genetic fingerprint can be of epidemiological value (Hilton et al., 1997). RAPD is a rapid and economical method that can be a very suitable tool in everyday practice and as a complement to traditional techniques such as serotyping (Ruiz et al., 2003). Although reproducibility is a major problem of the RAPD method, using a commercially available PCR buffer optimization kit in combination with methods to select thermal cycling parameters and discriminatory primers can improve the results (Hilton et al., 1997). Plasmid profiling and MLST can also be used as an adjunct to PFGE. Although plasmid profiling is a simple and inexpensive technique, plasmids are mobile elements that can be lost and acquired during outbreak/storage (Brown, 2005). Fakhr et al. (2005) compared the discriminatory power of PFGE to MLST in typing clinical isolates of *Salmonella enterica* serovar Typhimurium and concluded that MLST lacks the ability to discriminate between *S.* Typhimurium serovars and that PFGE can still be considered the method of choice for the molecular typing of this serotype.

Antimicrobial resistance profiling has also been used to characterize *Salmonella* isolates recovered from samples taken from farm environments (Nayak et al., 2004). Even

though the addition of antimicrobials to animal feed at sub-therapeutic levels can increase weight gain and feed efficiency while suppressing endemic disease, the impact of this practice on bacterial antibiotic resistance and its impact on human health is of great concern (Cox et al., 2003). Increasing antimicrobial resistance among *Salmonella* has been noted for several decades (Lee et al., 1994), particularly among strains of the serotype Typhimurium (Rabatsky-Ehr et al., 2004). Nayak et al. (2004) showed that the combination of PFGE, serotyping and antibiotic resistance profiling could better explain the transmission of antibiotic-resistant *Salmonella* strains within a turkey production facility.

1.3.6 Model Research Pathogen

Salmonella is an ideal foodborne pathogen model to study bacterial pathogenesis because it represents one of the best understood and studied microorganisms, therefore making it possible to design effective intervention steps based on defense mechanisms that the organism can potentially develop (Ricke et al., 2005). *Salmonella* Typhimurium has long served as a model organism for genetic studies with a wide variety of classical and molecular genetic tools available to identify and characterize potential *Salmonella* virulence genes (Ohl and Miller, 2001). The availability of *in vitro* tissue culture and small-animal infection models have facilitated its use in research. Furthermore, the intricacies of the host-pathogen interactions that determine the outcome of *Salmonella* infections has been extensively studied (Ohl and Miller, 2001). Previous studies have described many aspects of the organism's metabolism including important substrates and products (Batzing, 2002; Pommerville, 2004). Finally, a wide variety of techniques for isolating and identifying this organism have been published (Batzing, 2002; Pommerville, 2004). Therefore, in addition to

being one of the most problematic and resourceful pathogen, *Salmonella* has also served as a fairly useful model of foodborne pathogen research (Ricke et al., 2005).

1.3.7 Clinical Syndromes and Transmission

Human salmonellosis, especially those caused by nontyphoid *Salmonella*, can produce a variety of symptoms such as fever, diarrhea, cramping, abscesses, arthritis, bacteremia, and sepsis (Cherubin et al., 1969; Baird-Parker, 1990; Hohmann, 2001). Typhoid salmonellae, such as *Salmonella* Typhi and Paratyphi, cause a disease known as enteric or typhoid fever. Typhoid fever was extremely common at the beginning of the 20th century, however, with improvements in drinking water disinfection, sewage treatment, milk sanitation and pasteurization, and shellfish bed sanitation, the disease has practically disappeared in the United States (Tauxe, 1997). Generally, clinical signs of enteric infection with nontyphoid *Salmonella* can be indistinguishable from other enteric bacterial diseases although some distinctions can be made when a very bloody diarrhea occurs, which suggest *Shigella* or enterohemorragic *E. coli* (EHEC) infection (Hohmann, 2001). A wide spectrum of illness severity can occur which will depend on host immunity, inoculum size, bacterial virulence, and other factors. The majority of the human cases are either asymptomatic or gastrointestinal yet a small percentage of infections can develop into acute bacteremia and death (Cherubin, 1969). The salmonellosis death rate has been reported to be 1.5 deaths per 10,000,000 U. S. standard population for the year 2003 (Hoyert et al., 2006).

Some common risk factors for human salmonellosis are corticosteroid or antimicrobial drug use, cancer, diabetes, alteration of normal intestinal microflora, extremes of age, and immune system suppressant diseases such as HIV (Hohmann, 2001). These

factors also influence the incubation period which is generally 5-72 h, occasionally extending to 7 days (Baird-Parker, 1990). Although symptoms usually occur between 12 and 36 h post-inoculation and last from 2 to 5 days, the carrier state can last for more than 3 months (Baird-Parker, 1990). In addition, it has been reported that patients receiving antibiotic treatment were more likely to have both prolonged excretion and clinical relapse (Nelson et al., 1980; Baird-Parker, 1990). Results from animal trials support these findings by showing that antibiotic use suppressed the protective effect of the normal intestinal microflora, giving opportunity for the hardy *Salmonella* species to colonize and cause disease (Hohmann, 2001). Thus, antibiotic therapy is not routinely recommended for the empiric treatment of mild or moderate gastroenteritis caused by *Salmonella* in healthy individuals and management of clinical salmonellosis is usually limited to fluid replacement therapy.

Salmonella can infect humans through several routes including contact with colonized animals (zoonosis), food and water. Salmonellae are carried in the intestinal tract of most farm and wild animals including birds, reptiles, amphibians, and arthropods (Baird-Parker, 1990). Although *Salmonella* infections in animals are mostly asymptomatic, causing little or no disease in the hosts, the pathogen is usually excreted in large numbers in their feces (Baird-Parker, 1990). Contact with fecal matter of infected animals and man is an important source of contamination of the environment and the food production chain.

Salmonella can also infect and colonize poultry. Poultry salmonellosis can be divided in three categories: (1) nontyphoid *Salmonella* infections, (2) host-specific infections and (3) Arizoonosis. Generally, nontyphoid *Salmonella* infections, as in other animals, are asymptomatic but the pathogen can be present in large numbers in poultry feces and bedding material (Santos et al., 2005). Examples of nontyphoid *Salmonella* include *S.* Typhimurium,

S. Enteritidis and *S.* Heidelberg. These infections are of great concern because poultry is a significant source of *Salmonella* that can cause foodborne illness in humans (Hoszowski et al., 1996; Byrd et al., 1997). This group of pathogens can be transmitted to poultry by several routes including fecal-oral (Porter, 1998). Some other examples of vectors include feed, water, insects, rodents, poults, humans, and semen (Bailey et al., 2001; Donoghue et al., 2004). Moreover, a parent flock can transmit *Salmonella* to its progeny through the trans-ovarian route (Notermans et al., 1992; Ranta and Maijala, 2002). Intestinal colonization usually results in invasion of the intestinal wall and dissemination to internal organs (Brown et al., 1976). Although young birds are more susceptible to systemic infections resulting in increased mortality, adult birds are more resistant and may harbor *Salmonella* in their intestinal tract without showing clinical signs (Brown et al., 1976).

Similar to the human typhoid fever, there are some poultry diseases caused by *Salmonella* serotypes that are extremely adapted to the avian species. Although these infections are of little public health concern, they can cause major problems to the poultry industry, including high mortality and production losses. *Salmonella* Pullorum and *S.* Gallinarum are the ethiological agents of pullorum disease and fowl typhoid, respectively. These pathogens cause systemic disease in a wide range of domestic poultry including chickens, turkeys, ducks and other gallinaceous birds (Porter, 1998). Pullorum disease is most lethal in young birds of 3 weeks of age or less, with minimal effect on adults while the mortality observed in fowl typhoid affects young birds and persists into adulthood (Pomeroy and Nagaraja, 1991). Other signs such as respiratory distress, lameness, and blindness may occur depending on the immune system of the affected bird (Porter, 1998).

Arizoonosis generally occurs in young turkey poults with its etiologic agent being

Salmonella Arizona. Besides being horizontally transmitted (fecal-oral route and vectors), *S.* Arizona infection is also egg-transmitted. The most common symptoms of the disease are listlessness and trembling, pasting of the vent area, huddling near a light source, twisted head and neck, and cloudiness and enlargement of the eye causing blindness (Herenda et al., 2002). Necropsy of dead birds may reveal an enlarged yellow liver, congestion of the duodenum, unabsorbed yolk material, cheesy plugs in the intestine or ceca, inflammation of the oviduct and peritoneum, eye lesions, and purulent exudate in the brain (Herenda et al., 2002).

1.3.8 Statistics

The ERS report released in 2000 estimated that the total cost of foodborne illness in the U. S. is 6.9 billion dollars per year which includes losses due to medical costs, productivity losses and premature death associated with only 5 bacterial foodborne pathogens (USDA, 2004). The pathogens include non-typhoid *Salmonella*, *Listeria monocytogenes*, *Campylobacter* spp., *E. coli* O157:H7, and shiga-toxin producing *E. coli* non-O157 (STEC). According to the report, foodborne salmonellosis alone cost about 2.4 billion dollars per year which was the highest expenditure compared to the other four pathogens. The report also showed that of all salmonellosis cases, 0.04% were fatal and 1.16% of cases required hospitalization.

A total of 6,471 laboratory-confirmed cases of foodborne salmonellosis were identified in 2005, resulting in an overall incidence of 14.55 cases per 100,000 (CDC, 2006). The preliminary surveillance data for 2005 also shows that only six *Salmonella* serotypes accounted for 61% of the infections and included Typhimurium (19%), Enteritidis (18%),

Newport (10%), Heidelberg (6%), Javiana (5%), and a monophasic serotype identified as *Salmonella* I 4,[5],12:i:- (3%). Additionally, it was reported that the estimated annual incidence of *Salmonella* infections decreased 9% from 1996-1998 (baseline) to 2005. Although the overall incidence of *Salmonella* infections from these five serotypes declined, only the incidence of *S.* Typhimurium infections decreased significantly (42%); the estimated incidence of *S.* Enteritidis, Heidelberg and Javiana increased 25, 25 and 82%, respectively. The estimated incidence of infection by *S.* Newport increased compared to the baseline yet the increase was not statistically significant (CDC, 2006).

The three most common *Salmonella* serotypes isolated from human samples in 2004 (Typhimurium, Enteritidis, and Newport) accounted for 43% of the isolates, whereas the most common serotype found in broilers and turkeys was *S.* Heidelberg (from clinical sources), which accounted for 46.9 and 25.8 % of the poultry isolates, respectively (Figure 1). *S. Gallinarum* was isolated as frequently as *S.* Heidelberg from clinical samples of broilers. *Salmonella* Kentucky and Senftenberg were not influenced by the condition of the animal host (clinical or non-clinical) and was the second most often serotype isolated from broilers and turkeys.

The National *Salmonella* Surveillance System data on *Salmonella* isolates obtained from non-human sources can help identify possible sources of human illness. For example, *S.* Typhimurium, the most common serotype in humans, is identified most commonly from clinical samples derived from bovine sources and from non-clinical samples from chicken sources. In addition, *S.* Enteritidis and S. Heidelberg, the second and fourth most common serotypes in humans, respectively, are identified most commonly from clinical and non-clinical chicken sources (CDC, 2005a).

A large proportion of *S*. Typhimurium isolates were resistant to multiple antimicrobial drugs; in 2002, 21.1% of *S*. Typhimurium isolates characterized in the National Antimicrobial Resistance Monitoring System (NARMS) were resistant to one or more drugs and 30% had a five-drug resistance pattern characteristic of the commonly recognized phage type, DT104 (CDC, 2005a). In addition, *S*. Newport has emerged as a major multidrug-resistant pathogen. In 2002, 53 (23%) of 239 *S*. Newport isolates submitted to NARMS were resistant to at least nine of 17 antimicrobial agents tested, including extended spectrum cephalosporins (CDC, 2005a).

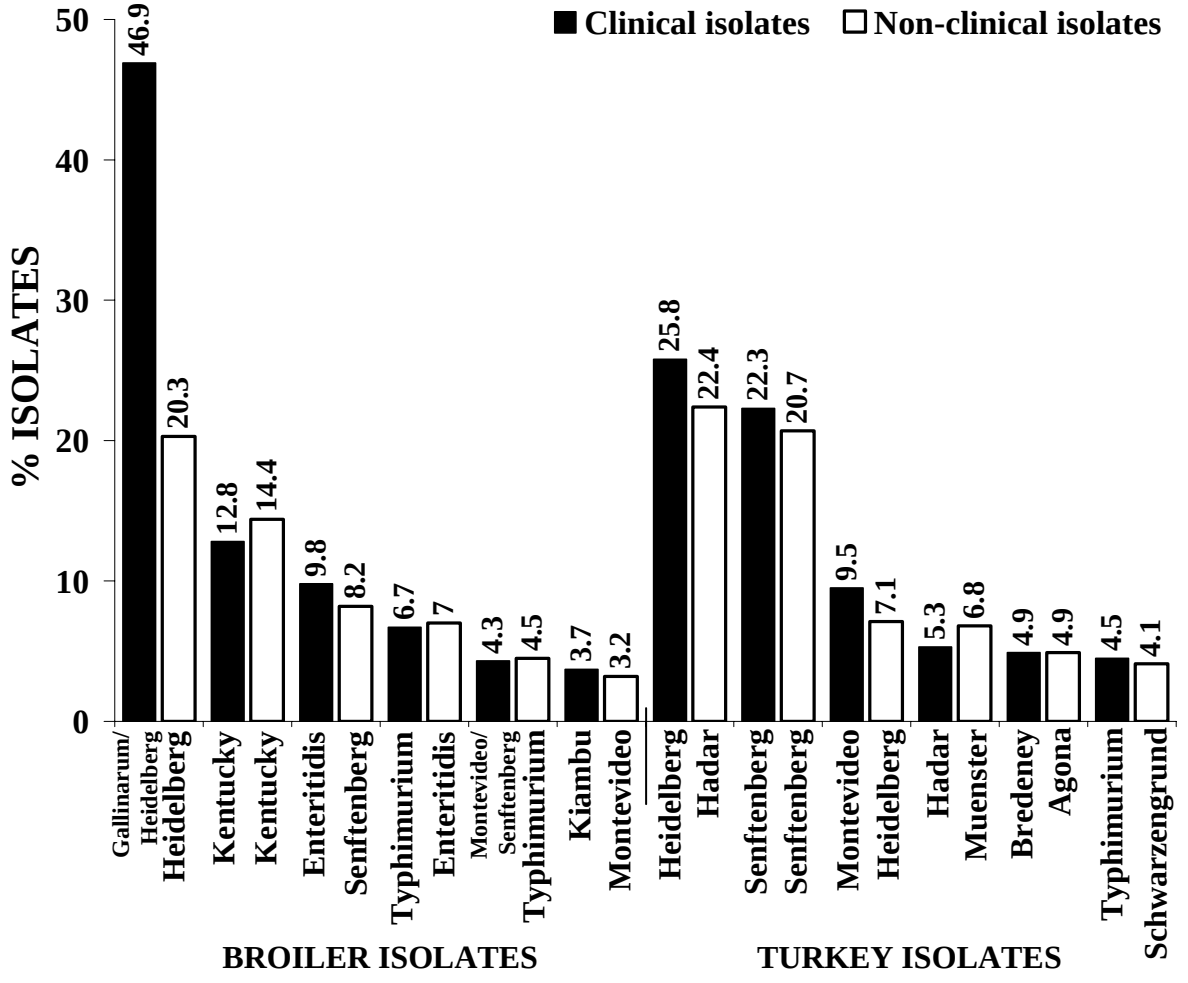

Figure 1. Percentage of clinical and non-clinical *Salmonella* serotypes isolated from broiler and turkey sources reported to CDC and NVSL in 2004 (Data source: *Salmonella* Annual Summaries, 2004 – CDC, 2005a).

1.4 PRE-HARVEST FOOD SAFETY

1.4.1 Management Strategies to Control Pathogen Colonization of Poultry

To control foodborne pathogen contamination of raw meat and poultry products at the processing level, the Food Safety and Inspection Service (FSIS) agency introduced the Pathogen Reduction, Hazard Analysis and Critical Control Point (HACCP) System in 1996 (USDA, 1996). The HACCP rule set *Salmonella* performance standards that should be met by all establishments that produce raw meat. The results of eight years of testing following introduction of HACCP revealed that the majority of processing establishments had met the performance standard. The progress report also showed that 86% of large plants, 73% of small plants and only 40% of very small plants met the *Salmonella* performance standards in 2005 (USDA, 2006b). Nevertheless, when product specific rates were further stratified by establishment size, broilers processed in very small plants exceed the baseline prevalence (20% positive) for the first time since 2001, with 33% positive *Salmonella* samples (USDA, 2006b). Additionally, over the past three years (2003–2005), significant increases in *Salmonella* rates were observed among poultry products (broiler carcasses, ground chicken and ground turkey). Certainly, the presence of *Salmonella* in poultry products at the time of inspection is just one factor contributing to the incidence of foodborne salmonellosis. A significant reduction or elimination of this pathogen will be achieved when effective interventions are applied to all segments of the food production and distribution chain including animal and plant husbandry, production, transportation, and preparation (USDA, 2006b).

Pre-harvest control of *Salmonella* represents a challenge to the poultry producer. It is well known that salmonellae can be introduced into a poultry flock through many vectors

including rodents, wild birds and insects. In addition, the fact that most birds will not develop any visual signs of *Salmonella* infection makes it even more difficult to control transmission of this pathogen. To worsen the situation, some serotypes of *Salmonella* can be transmitted from the breeder flock to its progeny via a vertical transmission route. *Salmonella* Gallinarum, *S.* Pullorum and *S.* Enteritidis have the capacity to colonize the hen's ovary and infect the egg prior to calcium deposition for eggshell formation. Additionally, Liljebjelke et al. (2005) reported that *S.* Typhimurium could also be transmitted through the transovarian route.

To control or eliminate *Salmonella* from poultry flocks, the animals must be kept under special pathogen-free conditions (Oosterom, 1991). This has many implications such as (a) the establishment of *Salmonella*-free breeding flocks; (b) the adoption of an "all-in-all-out" system; (c) the enforcement of strict hygiene practices on the farm, such as thorough cleaning and disinfection including clothing, facilities and vehicles; (d) the complete isolation of poultry houses from the environment, particularly with respect to wild birds, rodents, insects, and dust; (e) the wearing of special clothes and footwear; (f) the production of *Salmonella*-free feed; (g) the establishment of a safe water supply; (h) the use of *Salmonella*-free litter; (i) restricting entry to the farm to only essential workers and vehicles; (j) the restriction of pets on the farm; and (k) the required examination of diseased birds followed by laboratory diagnosis (Oosterom, 1991; USDA, 2003). Furthermore, it is necessary to implement additional hygiene measures during transport of the animals to the slaughter house and during the slaughtering procedures. Oosterom and Nothermans (1983) demonstrated that *Salmonella* contamination of swine can be reduced if strict hygiene measures are employed during slaughtering. Poultry producers are already familiar with strict

hygiene measures, as cleaning and disinfection of poultry houses is usually conducted every 6 weeks. Poultry hatcheries also follow strict hygiene practices including microbiological monitoring and disinfection of eggs before they leave the farm. However, *Salmonella* eradication programs should primarily include the establishment of *Salmonella*-free breeding flocks which can only be established by surveying the flocks and eliminating contaminated birds (Oosterom, 1991).

1.4.2 Nutritional Strategies to Control Pathogen Colonization of Poultry

Several experimental studies have been conducted to find ways to avoid or reduce *Salmonella* contamination in poultry processing and more recently during poultry production. Many of these initiatives have focused on the effect of specific feed components, such as probiotics and prebiotics, or on the addition of different organic acids (Bjerrum et al., 2005). In addition, several studies have shown the beneficial effect of growth-promoting antibiotics on growth performance; however, as a result of the increased concern over bacterial antibiotic resistance, the use of antimicrobial agents is not considered an acceptable method to eradicate salmonellae during production. In contrast to antibiotics, most natural feed additives do not reduce overall microbial loads. Instead, they alter the intestinal microflora profile by limiting the colonization of unfavorable bacteria and promoting the growth of favorable species. Beneficial effects of these alternative feed ingredients include change in GIT pH, maintenance of protective intestinal mucins, stimulating the growth of beneficial intestinal microorganisms, enhanced nutrient uptake, and increased humoral immune response (Ferket, 2003).

Altering dietary fiber (non-starch polysaccharides – NSP) levels by using alternative

cereals such as wheat or triticale and adding enzymes to the feed have been shown to influence intestinal microflora and promote intestinal health (Engberg et al., 2002; Engberg et al., 2004; Santos, 2006). The use of enzymes that hydrolyze NSP is now a routine practice of the feed industry, where they act to improve nutrient utilization and bird performance (Silva and Smithard, 2002; Engberg et al., 2004). These studies showed that enzyme supplementation in diets high in NSP content indirectly modifies the enteric microflora ecosystem, creating a more healthy state and reduced *Salmonella* colonization (Santos, 2006). Feed structure is also known to have some influence on salmonellae. It has been demonstrated that broilers given pelleted feed were more likely to carry *Salmonella* in their gizzards and ileum than birds supplemented with whole wheat (Bjerrum et al., 2005). These observations indicate that cereal-based diets high in NSP content, enzyme supplementation and feeding coarser diets promote intestinal health and exclude pathogens.

Probiotics and Prebiotics. A probiotic is defined as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). The major goal of this strategy is to promote the growth of groups of bacteria that are competitive with, or antagonistic to, pathogenic bacteria by filling all microbial ecological niches and thereby preventing the establishment of an opportunistic pathogenic bacterial population. Various probiotic techniques involve introducing a beneficial microbial population to the GIT or providing a limiting nutrient (also known as prebiotic) that allows an existing commensal microbial population to expand its role in the GIT (Callaway et al., 2003). Therefore, prebiotics are nutrients that are not digested by the host, but they benefit the host by selectively stimulating the growth and activity of one or a limited number of

bacterial populations in the GIT, predominantly those that produce short-chain fatty acids (SCFA).

Lactobacillus and *Bifidobacterium* species have been used most extensively as probiotics in humans whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock (Salminen et al., 1998). Probiotic microorganisms inhibit growth of potentially pathogenic microorganisms by competitive exclusion (CE) or the so-called "Nurmi concept" (Nurmi and Rantala, 1973). The commensal microflora competitively exclude pathogens by lowering the pH through production of acids and SCFA (Fuller, 1977), competing for niches and available nutrients (Hungate, 1966), producing bacteriocins (Jack et al., 1995), stimulating the intestinal associated immune system through cell wall components (Nousiainen and Setala, 1998), and stimulation of intraepithelial lymphocytes and natural killer cells (Ishizuka and Tanaka, 2002; Ishizuka et al., 2004). Although the use probiotics have resulted in improvement in performance, decreased mortality, and better feed conversion ratios (FCR) in poultry, they have not always been commercially adopted due to the use of other non-complimentary strategies such as sub-therapeutic antibiotic use (Callaway et al., 2003). Considering that the usage of growth-promoting antibiotics is declining, probiotic strategies are becoming more effective and more widely accepted by the poultry industry.

The use of prebiotics has several advantages over probiotics where culture viability needs to be maintained. Several commensal bacterial communities that are present in a "healthy intestine" cannot be cultured so they cannot be used in commercial probiotic products. However, dietary supplementation of sugars and other organic compounds that are not digested by the host has been shown to stimulate these noncultivable bacteria in humans

(Rastall et al., 2005), dogs (Willard et al., 2000) and swine (Konstantinov et al., 2003). Additionally, prebiotics are more stable to the heat and pressure used during feed manufacturing. Prebiotics also have an economical advantage because most of them are derived from inexpensive food processing by-products (Playne and Crittenden, 1996).

Fructoologosaccharides and galactoologosaccharides are some examples of sugars that are not degraded by intestinal enzymes and therefore pass down to the cecum and colon where they serve as substrates for bacterial fermentation (Willard et al., 2000). It has been reported that oligosaccharides and polysaccharides are preferentially utilized by *Bifidobacteria* (Yazawa et al., 1978). Fermentation of these sugars results in the increased lactic acid and SCFA production in the ceca leading to a reduction in the GIT pH, a similar effect caused by organic acids on susceptible gram-negative organisms. Swanson (2002) observed that prebiotics affect the immune function of humans and dogs by stimulating lactic acid bacteria. The rise in intestinal lactic acid bacteria populations stimulate phagocytic activity (cellular immune response) or IgA secretion (humoral immune response) or both.

Coupling the use of probiotics and prebiotics, known as symbiosis, could yield a positive synergistic effect in the reduction of foodborne pathogenic populations in food animals (Callaway, 2003).

Acidifiers and Organic Acids. Acidifiers and organic acids have been routinely used in the feed milling industry to preserve and protect feed from microbial and fungal spoilage or to increase the preservation effect of fermented feeds (e.g. silages). Organic acids have also been used as S*almonella*-control agents in feed and water supplies for livestock and poultry due to their strong bacteriostatic effects (Ricke, 2003). The most common organic

acids used in animal nutrition are citric, propionic, fumaric, lactic, formic and benzoic acids (Russell, 1992).

Although organic acids are often applied to the litter to control pathogen contamination, they are seldom used as feed additives because limited positive responses in weight gain and feed conversion have been observed (Langhout, 2000). However, dietary supplementation of fumaric, propionic, sorbic and tartaric acids has been shown to improve feed conversion ratio and growth of broilers (Vogt et al., 1982). Organic acids have mainly been used to sanitize the feed and more recently to reduce *Salmonella* colonization in poultry (Iba and Berchieri, 1995; Thompson and Hinton, 1997). Generally, lactic acid bacteria are able to grow at relatively low pH which means that they are more resistant to organic acids than other bacterial species such as *E. coli* and *Salmonella*. Lactic acid bacteria, like other gram-positive bacteria, have a high intracellular potassium concentration which counteracts the presence of acid anions (Russell and Diez-Gonzalez, 1998).

As with antibiotics, continued use of acidifiers and organic acids may result in the development of resistance among some pathogenic bacteria. Adaptation or tolerance to acidic environments, as discussed earlier, is recognized as an important survival strategy for many microorganisms. In addition, different microorganisms have developed different acid survival strategies. The acid resistance response of *Salmonella* Typhimurium to exposure to SCFA in the gastrointestinal tract of a host animal or in a food product might also cause enhancement in the virulence of the pathogen (Kwon and Ricke, 1998).

Alternative Cereals and Grain Particle Size. The use of grains other than corn has

become a common practice, especially in European countries (Hetland and Svihus, 2001; Svihus et al., 2004). Moreover, cereals can also be added as whole grains to poultry diets resulting in improvement in growth performance of broilers, turkeys, and layers (Bennett et al., 2002a; Bennett et al., 2002b; Lázaro et al., 2003; Svihus et al., 2004). Besides improvements in bird growth, another benefit of whole grain feeding is reduced feed cost as a result of reduced handling and processing (Svihus et al., 2004).

Cereals such as oat, barley, wheat, rye and triticale are rich in fiber, also known as non-starch polysaccharides (NSP). NSP are heterogeneous groups of polysaccharides with varying degrees of water solubility, size, and structure; they represent the principal component of the kernel that is not digested by endogenous secretions in the digestive tract (Lineback and Rasper, 1988). Some of the beneficial impacts of structural carbohydrate components of NSP have been used for many years in poultry diets and more recently they have been examined as potential prebiotics (Santos, 2006). As an example, beta-glucan (β-glucan) was shown to stimulate microbial fermentation, modulate immunity by increasing mammalian macrophage and neutrophil activity *in vitro* (Kataoka et al., 2002), and to protect broiler chickens against *Salmonella* challenges by up-regulating heterophil phagocytosis, bactericidal killing and oxidative burst (Lowry et al., 2005). Additionally, reduction of some gram-negative bacteria, such as coliforms, and increase of bifidobacteria and lactobacilli, which are potentially health-promoting bacteria, was reported in human studies after supplementing diets with rafinose (galactooligosaccharides) (Matteuzzi et al., 2004). Another highly investigated indigestible sugar is galactomannan which is extracted from partially hydrolyzed guar gum. Galactomannans has been reported to reduce diarrhea (Takahashi et al., 1993), improve the intestinal microflora ecology in humans (Okubo et al., 1994) and to

suppress the multiplication of *Salmonella* Typhimurium *in vitro* (Oyofo et al., 1989) and in laying hens (Ishihara et al., 2000). Additionally, galactomannan supplementation in laying diets was shown to increase *Bifidobacterium* spp. and *Lactobacillus* spp. populations and to decrease colonization of *Salmonella* Enteritidis (Ishihara et al., 2000). Arabinoxylans have also been reported to stimulate microbial fermentation and to activate a macrophage cell line in the broiler intestine thereby decreasing enteric pathogen colonization (Zhang et al., 2004). Dietary fiber is also generally accepted as having protective effects against a range of human diseases including colorectal cancer, coronary heart disease, diabetes, obesity, and diverticular disease (Chaplin, 2006).

It is commonly understood among animal nutritionists that reducing particle size of grains improves pellet quality and nutrient utilization by increasing the surface area of the feedstuff. This in turn increases the exposure to digestive enzymes leading to an improvement in overall nutrient digestibility. However, some studies have shown that whole wheat feeding improves digestion of energy containing nutrients (Svihus et al., 2004) and weight gain of broilers (Plavnik et al., 2002). Similar results were observed in broilers (Bennett et al., 2002a) and turkeys (Bennett et al., 2002b) fed whole barley.

Whole grain feeding has been demonstrated to alter intestinal microflora composition and to reduce *Salmonella* colonization in broilers. For example, whole wheat feeding has been shown to reduce *Salmonella*-positive samples of gizzard contents and *Salmonella* Typhimurium populations in ileal contents (Bjerrum et al., 2005). Similarly, *Salmonella* death rates in the gizzard contents of broilers fed fine mash or pelleted diets was reduced when compared to coarser mash feeding (Huang et al., 2006). Engeberg et al. (2004) reported that broilers fed whole wheat had a higher number of lactobacilli in the gizzard and reduced

lactose-negative bacteria and enterococci throughout the GIT. The authors also reported that the growth of *Clostridium perfringens* tended to be inhibited in the intestinal contents following whole wheat feeding, which was later confirmed by Bjerrum et al. (2005) who reported a large reduction of *Clostridium perfringens* populations following whole wheat feeding. Clostridiosis or necrotic enteritis is of great concern to the poultry industry because this disease causes significant economic losses due to poor bird performance and mortality. The reason why whole wheat feeding inhibits intestinal growth of *Clostridium perfringens* is still unknown. A possible explanation for the inhibition may be related to the improved function of the gizzard following whole grain feeding. This in turn regulates the filling of the small intestine which may result in lower concentrations of easily fermentable nutrients such as starches, making it more difficult to bacteria such as *Clostridium perfringens* to proliferate (Bjerrum et al., 2005).

Another benefit of alternative cereals, other than improvements in bird performance, include reduced feed cost as a result of reduced handling and feed processing. Wondra et al. (1995) showed that milling energy increased and production rate slightly decreased after reduction of corn particle size from 1,000 to 600 microns when utilizing a hammermil. Moreover, the energy required to reduce particle size from 600 to 400 microns was more than twice that required to achieve 600 microns and production rate also decreased significantly. Additionally, the type of grain milled also affects energy usage and production rates. Less energy is required to grind sorghum to 500 microns than to grind corn to 900 microns (Healy et al., 1994). Furthermore, Baker (1960) reported that sorghum grain was easier to grind than corn and corn was easier to grind than oats.

Enzymes. Enzymes are important catalysts that can initiate or accelerate the speed of reactions occurring in a living organism or in the environment. Even the simplest living organisms contain multiple copies of nearly a thousand different enzymes (Horton et al., 1996). Recently the commercial use of isolated enzymes has become a routine practice in the poultry industry for improving nutritional value of feed, especially in European countries where wheat has been used as the main ingredient in poultry diets.

Since the 1920s researchers have observed beneficial effects from enzyme supplementation in poultry feeds, particularly feeds with high fiber content (Hastings, 1946; Moran and McGinnis, 1968; Engberg et al., 2004; Hogberg and Lindberg, 2004). Supplementing feed with enzymes is done to alleviate the adverse effects of antinutritional factors (Santos, 2006), render certain nutrients more available for absorption and enhance the energy value of feed ingredients (Lyons and Walsh, 1993), and modulate intestinal microflora to a healthier state (Engberg et al., 2004; Hogberg and Lindberg, 2004).

The major enzymes used in animal feeds are proteases, amylases, lipases, phytases, NSP-degrading enzymes, and cellulases. Additionally, hydrolases are recommended for use in animal husbandry to improve dietary nutrient utilization (Modyanov and Zel'ner, 1983). Commercial enzyme products are typically a blend of several different enzymes that are effective on a wide variety of substrates. The enzymes with proven efficacies for animal husbandry include xylanase, arabinoxylanase, β-glucanase, cellulase, and phytase (Choct and Kocher, 2000; Santos, 2006).

Non-starch polysaccharide-degrading enzymes are now commonly used in cereal-based diets for poultry. It is well documented that most fungal and bacterial preparations effectively degrade viscous polysaccharides (e.g. β-glucan, arabinoxylan) present in barley,

oats, rye and wheat (Jensen et al., 1957, Odetallah et al., 2002; Silva and Smithard, 2002). Since chickens and turkeys do not produce enzymes that are capable of digesting xylans and β-glucans, exogenous NSP enzymes are typically added to their feed (Silversides and Bedford, 1999). Xylanase supplementation improves nutrient availability because these enzymes degrade the xylan backbone of arabinoxylan into smaller units which results in several beneficial results, including increased availability of xylose units for monogastrics (Odetallah, 2000), disruption of water holding capacity of the NSP (Scott and Boldaji, 1997), and reduction of digesta viscosity in the small intestine (Choct et al., 1999; Santos, 2006). Reduced digesta viscosity increases the diffusion rates of nutrients and endogenous enzymes, enabling the bird to better digest and absorb more nutrients (Pawlik et al., 1990). Endoxylanase supplementation also inhibits the proliferation of fermentative microorganisms in the small intestine by increasing the digesta passage rate and nutrient digestion (Choct et al., 1999). Thus, nutrient utilization is improved by reducing the competition between the host and its enteric microflora. Although microbial fermentation in the small intestine decreases when xylanases are added to the diet, microbial fermentation increases in the large intestine and ceca (Steenfeldt et al., 1998). Steenfeldt et al. (1998) observed a decrease in the pH of the cecal contents of chickens as a result of enzyme supplementation. The decreased pH was the direct result of the production of a higher concentration of SCFA due to increased microbial fermentation, demonstrating that the degradation of arabinoxylan in the enzyme-supplemented diets increases the amounts of nutrient available for microbial fermentation in the ceca. Increasing levels of short-chain fatty acids increases the nutrient value of the diet because they are readily absorbed and utilized by the bird to generate energy (Choct et al., 1996).

In conclusion, supplementation of diets rich in NSP with NSP-enzymes has been shown to improve the performance of poultry by improving digestion, absorption of nutrients, and modulating intestinal microflora (Santos, 2006).

Antibiotics. Antibiotics have been used worldwide in agriculture to promote the growth and welfare of animals for about 50 years (Dibner and Richards, 2005). Many of these antibiotics are given at sub-therapeutic dosages and are commonly called growth-promoting antimicrobials (GPA). The beneficial effects of antibiotic supplementation on production efficiency of poultry and swine were reported in the late 40's and early 50's (Moore et al., 1946; Jukes et al., 1950). However, the use of antimicrobials in food animal production has become highly controversial in recent years due to the potential development of antibiotic-resistant bacteria (Swartz, 2002; Threlfall, 2002; Woodward, 2005). Resistance among gram-negative bacteria such as *E. coli* and *Salmonella* has generated strong objection to antibiotic use (Gustafson and Bowen, 1997).

In spite of these potential drawbacks to subtherapeutic antibiotic treatment, recent research has found that some antibiotics do have the potential to improve food safety at the live animal level (Callaway et al., 2003). Neomycin sulfate is an antibiotic approved for use in cattle and has a 24-h withdrawal period. In a study by Elder et al. (2002), cattle fed neomycin for 48 h followed by a 24-h withdrawal period shed significantly lower generic *E. coli* and *E. coli* O157:H7 populations in their feces. After 5 d of neomycin withdrawal, generic *E. coli* populations had returned to near pretreatment levels, yet *E. coli* O157:H7 populations remained nearly undetectable (Elder et al., 2002). Other antimicrobial compounds are still incorporated into animal diets to improve animal health and growth

performance. Ionophores are antimicrobials used in human medicine but not related to antibiotics and therefore do not appear to lead to an increase in antibiotic resistance (Callaway et al., 2003). Monensin, the most widely used ionophore, has been used both as a coccidiostat in poultry and as a growth promoter in ruminants (Russell and Strobel, 1989). Because ionophores are potent antimicrobials that are approved for use in food animals, it was assumed that they could be used to control food-borne pathogens. Unfortunately, because of the physiology of some common foodborne pathogens, it does not appear that ionophores reduce food-borne pathogenic bacteria populations (Busz et al., 2002).

1.4.2 Bacterial Antibiotic Resistance

One of the first reports of antibiotic resistance in food animals was made by Starr and Reynolds (1951) after experimental feeding of streptomycin to turkeys. Even though the addition of antimicrobials to animal feed at sub-therapeutic levels (growth-promoting antimicrobials) can increase weight gain and feed efficiency while suppressing endemic disease, the impact of this practice on increasing bacterial antibiotic resistance and its impact on human health is of great concern (Cox et al., 2003). Increasing antimicrobial resistance among *Salmonella* has been noted for several decades (Lee et al., 1994), particularly among strains of the serotype Typhimurium (Rabatsky-Ehr et al., 2004). Early concerns about the development of antibiotic resistance in human pathogens and recommendations to ban sub-therapeutic use in animal feed were discussed by Swann in a report to the British Parliament (1969). The long term and extensive use of antibiotics in human and veterinary medicine has resulted in the selection of resistant bacterial strains. Genes encoding such resistance may be transferred to other formerly susceptible bacteria, thus posing a threat to both animal and

human health (Montagne et al., 2003).

In 1997 the European Union (EU) banned the use of most antibiotics as feed additives or for use in sub-therapeutic dosages in food-producing animals (Cervantes, 2005). More recently the European Commission's Standing Committee on the Food Chain and Animal Health (SCFCAH) approved a proposal for a regulation that targets the reduction of the incidence of the five most common *Salmonella* serotypes in chicken breeding flocks (USDA, 2005). The regulation allows only two recommended antibiotic usages: to rebuild a new flock from the hatching eggs of an infected flock, or therapeutically for birds with clinical signs of salmonellosis.

A review of the impact of the ban of antibiotic usage in the EU revealed that a significant decrease in antibiotic resistance among bacteria isolated from raw meat products had occurred (Cervantes, 2005). However, the author indicated that the decrease in antibiotic resistant-bacteria isolated from raw meats had not translated into lower levels of antibiotic resistance in human patients. Another adverse consequence of the European ban of GPA for the poultry industry has been a rising incidence of colibacillosis and necrotic enteritis (Truscott and Al-Sheikhly, 1997) and a decrease in performance, nutrient uptake, intestinal health, and profitability of livestock production (Lovland and Kaldhusdal, 2001; Casewell et al., 2003; Cervantes, 2005). Additionally, an increase in enteric diseases in livestock production in Denmark was followed by a considerable increase in antibiotic use for therapeutic or veterinary purposes (DANMAP, 2004). The veterinary consumption of prescribed antimicrobials increased from 48,000 kg in 1996 to 112,500 kg of active antimicrobial compounds in 2004. This represented a 135% increase in antimicrobial use for veterinary purposes over 8 years. Moreover, the therapeutic antibiotics used to treat the food-

producing animals were among the same classes of antibiotics most frequently used in human medicine (Cervantes, 2005).

Certainly, the period between the abolishment of an antibiotic and the reduction of bacterial resistance to the drug varies considerably from drug to drug (Swartz, 2002). Generally, this interval is only a few years as was the case for avoparcin use on Danish farms where the prevalence of resistance among *E. faecium* declined from 82 to 9% in only 2 years. Even after a marked decline, glycopeptide resistant *Enterococcus faecium* recovered from broilers and pigs in Denmark could still be found 6 years after the ban (Aarestrup et al., 2001). Despite all the controversy, it is now well recognized that prolonged exposure to antimicrobials will inevitably result in bacterial resistance (Swartz, 2002; Cervantes, 2005). Therefore, continuous efforts are necessary to develop new approaches to limit the colonization of poultry with foodborne bacterial pathogens while maintaining or improving productivity. In addition, on-farm monitoring programs for bacterial resistance can be helpful in recognizing the spread of resistance. (Swartz, 2002).

1.5 CURRENT STUDY FOCUS

Salmonella is an enteric pathogen that has been closely associated with poultry products (Hoszowski et al., 1996). Past studies have demonstrated that contamination of poultry can occur at any point in the production continuum (Bains and MacKenzie, 1974; Bryan and Doyle, 1995). Numerous studies have determined the incidence of *Salmonella* contamination during the grow-out phase (Read et al., 1994; Nayak et al., 2003), while few studies have actually measured *Salmonella* populations because of time, methodological and cost considerations. Control of *Salmonella* at the pre-harvest level could help to reduce

contamination prevalence and populations in the farm environment and at the processing plant. Therefore, the studies described in this dissertation were aimed at investigating alternative ways to reduce *Salmonella* contamination on and in the bird during the pre-harvest phase. It was hypothesized that *Salmonella* prevalence and populations are still highly present on commercial poultry farms and the use of different management practices such as housing design and dietary manipulations including altering grain particle size or fiber composition of the feed can reduce *Salmonella* colonization of poultry.

In the first chapter, the review of the literature focused on *Salmonella*, salmonellosis and the impact of this infection on human life and poultry production. Strategies used to prevent both poultry disease and pathogen colonization of the avian GIT and ways to improve growth performance and consequently increase profitability were also addressed. Additionally, concepts required for maintaining intestinal health with an emphasis on some nutritional strategies to modulate microbial ecology to discourage the colonization of unfavorable bacterial communities, including the possible effects of non-starch polysaccharides (fiber) and enzyme supplementation were reviewed. Lastly, bacterial antimicrobial resistance issues were briefly discussed.

The objective of the first experiment, described in Chapter 2, was to estimate *Salmonella* populations and prevalence in fresh excreta and litter obtained from commercial North Carolina turkey farms as a function of farm, season, and bird age. To test the hypothesis that high *Salmonella* populations and prevalence are present in commercial turkey farms and are influenced by season, farm and turkey age, a total of 12 commercial turkey farms were surveyed across two seasons (summer and winter) and two ages (3 weeks representing the brooding phase and 19 weeks representing the finishing/grow-out phase).

In order to characterize the *Salmonella* isolates recovered from fecal and litter samples taken from these commercial turkey farms, an experiment was conducted and the results are summarized in Chapter 3. *Salmonella* isolates were characterized by serotyping using the Kauffman-White Scheme, genotyping using pulsed-field gel electrophoresis, and antibiotic resistance/susceptibility profiling using gram-negative susceptibility plates (Sensititre) in order to test the hypothesis that *Salmonella* genotypes, serotypes, and antibiotic resistance profiles of commercial turkey farms are influenced by season, farm and turkey age.

An additional objective was to study different dietary manipulations and management practices that have the potential to reduce *Salmonella* colonization of poultry. Specifically, the purpose of experiment 3 was to determine the effect of inclusion of wood shavings and coarse ground corn on cecal *Salmonella* colonization (Chapter 4). Furthermore, the differences in *Salmonella* populations were also compared to changes in growth performance, structural characteristics of the small intestine, and pH of the upper gastrointestinal tract of turkeys fed corn-SBM diets. It is believed that the use of coarse ground corn or wood shavings (as a source of insoluble fiber) can improve gizzard development and activity and increase acid secretion in the pro-ventriculus, factors that may improve digestion and reduce *Salmonella* cecal colonization of turkeys. Similarly, in Chapter 5 the effects of feeding whole grains and housing design on cecal *Salmonella* colonization of broilers were studied. Additionally, the shift in *Salmonella* populations was associated with changes in growth performance, structural characteristics of the small intestine, and carcass yields of broiler chickens fed triticale- or corn-SBM diets.

Finally, to demonstrate that the effects of housing design, use of alternative NSP-rich cereals and different feed particle size on *Salmonella* colonization and growth performance

of broilers are associated with changes in bacterial population of the GIT, a study was conducted where the ileal bacterial populations were analyzed using DGGE to separate PCR amplicons of the 16S rDNA gene (Chapter 6). Results suggested that increasing the dietary fiber content of a corn basal diet by replacing it with triticale may change the intestinal microbial community diversity and hence discourage *Salmonella* colonization through the process of competitive exclusion. Finally, results of these five experiments are discussed and summarized in a summary chapter (Chapter 7).

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CHAPTER 2

ESTIMATION OF MOST PROBABLE NUMBER *SALMONELLA* POPULATIONS FROM COMMERCIAL NORTH CAROLINA TURKEY FARMS[*]

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Journal of Applied and Poultry Research

[*] Use of trade names in this publication does not imply endorsement by the North Carolina Agriculture Research Service or the North Carolina Cooperative Extension Service of the products mentioned, nor criticism of similar products not mentioned.

2.1 SUMMARY

Salmonellae are one of the primary causes of human gastroenteritis in the United States. Although there are many foods that may be contaminated with *Salmonella*, poultry products are one of the major vehicles for transmitting this organism to humans. However, the national incidence of poultry product contamination with *Salmonella* has declined since adoption of the HACCP food safety program. Further reductions in carcass contamination may require other approaches such as the adoption of on-farm pathogen reduction strategies. In this study *Salmonella* prevalence and populations from fresh excreta and litter composite samples taken from 12 commercial turkey farms were enumerated using the most probable number (MPN) method and compared as a function of farm, season (summer and winter), and bird age (3 vs 19 wk). Moreover, litter pH, temperature, moisture content, water activity, and ammonia levels were monitored. All farms were *Salmonella*-positive for at least one of the seasons, with populations ranging from <1 log MPN/g to >5.3 log MPN/g. Of the 48 separate fecal and litter composite samples analyzed, 70% and 79% were *Salmonella*-positive, respectively. Although the MPN enumeration method is much more labor intensive and costly than the prevalence method, it yields estimates of *Salmonella* populations instead of merely indications of presence or absence of the organism. Moreover, our findings demonstrated that the MPN method is significantly more sensitive compared to the prevalence procedure (for fecal samples). This study also demonstrated that *Salmonella* can be present at high populations during turkey production and that their populations and prevalence were significantly impacted by flock age (litter) and season by farm interactions (fecal). Furthermore, litter *Salmonella* populations appear to be associated with the interrelated parameters of litter pH, ammonia and moisture content.

Keywords: *Salmonella* populations, turkeys, farm, , litter, feces

2.2 DESCRIPTION OF PROBLEM

Contaminated poultry carcasses remain an important concern to the poultry industry, government agencies, and the public. To control or eliminate these potential food safety hazards, individual Hazard Analysis Critical Control Point (HACCP) food safety programs have been developed and implemented in all U.S. processing plants under Federal inspection. This Federally-mandated program calls for the testing of poultry carcasses and products for the presence of the foodborne pathogen *Salmonella* and generic *E. coli* and conducting lethality, stabilization, and *Listeria monocytogenes* testing for validating a processor's cooking, cooling, and post-process handling procedures. Processors must assure control of *Salmonella* by the use of such processing procedures as bird washers and chillers, although they are not held responsible for complete pathogen elimination on uncooked products since contaminated birds often arrive at plants with either undetectable to heavy pathogen loads. In these cases, the use of proper in-plant control procedures to reduce or eliminate this pathogen may not always be effective in assuring compliance with the processor's HACCP plan and Federal *Salmonella* performance standards. It is apparent that the implementation of other on-farm pathogen reduction strategies by poultry integrators (i.e., hatchery, feedmill, breeder and grow-out operations) may be necessary to successfully control pathogens and comply with Federal in-plant standards.

Salmonella is an enteric pathogen that has been closely associated with poultry products (Tauxe, 1991; Hoszowski et al., 1996; Byrd et al. 1997, Cox et al., 2000).

Salmonella and other pathogens can be commonly found in wastes such as human sewage, farm effluents, poultry litter, and other types of materials containing fecal matter (Ashton, 1990). Each year in the U.S., *Salmonella* is responsible for an estimated 300,000 to 4,000,000 cases of foodborne illness (USDA, 1998) and 30.6% of the deaths from foodborne illness (Kiessling et al., 2002). Salmonellae not only cause human illnesses and deaths, but cost consumers and U.S. businesses millions of dollars in annual medical treatment costs and lost productivity (Bryan and Doyle, 1995).

Previous studies have demonstrated that contamination of poultry can occur at any point in the production continuum (Bains and MacKenzie, 1974). Control of *Salmonella* at the pre-harvest level could help to reduce the contamination incidence and populations at the processing plant. It is anticipated that when contamination levels are reduced in and on the live bird, the risk of cross-contamination of carcasses and the finished product during processing should also be reduced. Before HACCP-type programs are extended to the grow-out phase, considerable research must first be conducted to assess the current populations and prevalence of pathogens present on the farm and to identify critical control points that effectively reduce or eliminate *Salmonella*. While numerous studies have determined the incidence of *Salmonella* contamination during the grow-out phase (Read et al., 1994; Nayak et al., 2003), very few studies have actually measured *Salmonella* populations because of time and cost considerations. Quantitative studies related to ventilation and populations of enteric pathogens on broiler chicken farms have been reported (Mallinson et al., 2000; De Rezende et al., 2001). The following study was conducted to estimate *Salmonella* populations, as a function of farm, season, and bird age, in fresh excreta and litter from commercial North Carolina turkey farms. Additionally, litter pH, temperature, moisture

content (MC), water activity (a_W) and ammonia levels were also measured and compared to the pathogen data.

2.3 MATERIALS AND METHODS

2.3.1 Experimental Design

Salmonella populations were estimated in fecal and litter samples taken from two houses per farm across twelve commercial turkey farms and two seasons, winter (November through March) and summer (April through October). Each farm consisted of houses containing curtain-sided ventilation, clay-lined floors, and pine shavings serving as bedding material. All brooder and grow-out farms followed similar management practices since they were contracted by the same parent integrator company. Moreover, litter was removed and houses cleaned and sanitized between flocks. The farms were coded as F1 through F6 for 3 week-old birds (brooder farms) and F7 through F12 for 19 week-old birds (grow-out farms). Because of logistical and integrator policies, no attempt was made to follow the same flock from the brooder phase through the grow-out phase of production.

2.3.2 Sampling Procedures

Litter samples measuring approximately one cubic inch (2.54 cm^3) and weighing approximately 10 g each were aseptically taken from 10 points in between the water and feed lines following a zigzag pattern throughout each house. The 10 samples were pooled in a Ziploc bag and stored on ice during transport to the laboratory. Fresh composite fecal droppings were also sampled across the entire length of the house, pooled together in sterile Whirl-Pak bags (Fisher Scientific Int., Bohemia, NY), and stored on ice in a transport cooler.

Litter temperatures were measured at 10 points in between the water and feed lines (the same depth and locations where litter samples were taken for microbiological analysis) using a calibrated traceable dual-channel thermometer (Fisher Scientific Int., Bohemia, NY) and an iron thermocouple probe (Omega Engineering Inc., Stamford, CT). Litter ammonia concentrations were measured at floor level (Pope and Cherry, 2000) from 3 locations across the house (both ends and middle) using 5 to 70 ppm capacity short-term tubes (Fisher Scientific Int., Bohemia, NY) and an ammonia gas detector pump (Drägerwerk Aktiengesellschaft, Germany). A 1.6 L capacity flower pot (with a small opening in its base) was placed up-side down on the litter where it remained for a 5-minute equilibration time. The short-term tube was subsequently inserted in the opening and ammonia readings were measured following procedures as recommended by the manufacturer. Ammonia test tubes were used once and discarded.

2.3.3 Most Probable Number Technique

Upon arriving at the laboratory, 25 g of each composite sample were placed in separate 7 x 12 inch (17.78 x 30.48 cm) sterile filtered stomacher bags (Spiral Biotech Inc., Norwood, MA) followed by the addition of 50 ml of buffered peptone water (BPW, Oxoid Ltd., Ogdensburg, NY) to each bag. The bags were then homogenized for one minute using a stomacher (Moriñigo et al., 1986; Wiberg and Norberg, 1996).

A three tube most probable number (MPN) technique was employed using BPW as a pre-enrichment broth (Moriñigo et al., 1986; Sinell et al., 1990; Tate and Miller, 1990; Davison et al., 1995; Dufrenne et al., 2001; Voogt et al., 2001). Ten milliliters were taken directly from each bag, placed into empty sterile test tubes (identified as 10^0 dilution), and

then 1 ml of sample was transferred to triplicate 9 ml BPW dilution tubes followed by serial dilution in triplicate tubes of BPW. All tubes were then incubated at 37°C for 18 to 24h (Moriñigo et al., 1986; Dufrenne et al., 2001) before transferring 0.1 ml of the appropriate dilutions to triplicate tubes containing 10 ml of Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Ogdensburg, NY) for selective enrichment (Moriñigo et al., 1986; Sinell et al., 1990; Dufrenne et al., 2001; Voogt et al., 2001). All RV broth tubes were incubated at 42°C for 24 h (Sinell et al., 1990; Dufrenne et al., 2001). Following incubation, one loopful from each tube was streaked for isolation onto modified lysine iron agar (MLIA, Oxoid Ltd., Ogdensburg, NY) (Cox et al., 2000) and incubated at 37°C for 24 h. Suspect colonies were picked, streaked, and stabbed onto triple sugar iron (TSI, Difco, Sparks, MD) agar slants (Cox et al., 2000) and then incubated at 37°C for 24 h. Presumptive positive *Salmonella* colonies were confirmed by agglutination using poly-O antiserum (Difco, Sparks, MD) (Wiberg and Norberg, 1996; Cox et al., 2000). Populations of *Salmonella* for each sample were determined using the Thomas' approximation (Blodgett, 2001; Swanson et al., 2001).

2.3.4 Salmonella Prevalence

For determining *Salmonella* prevalence, 25 g of each sample were placed in a sterile filtered stomacher bag (Spiral Biotech Inc., Norwood, MA) containing 100 ml of lactose broth (LB) (Difco, Sparks, MD) and stomached for one minute. An additional 125 ml of LB was added to the homogenized samples resulting in a 1:10 dilution (Beli et al., 2001; Andrews and Hammack, 2003). The bags were mixed for an additional 60 seconds and then incubated for 18 to 24 hours at 37°C. Similar to the MPN technique, a selective enrichment step was performed by transferring 1 ml from each bag to a bottle containing 100 ml of RV

broth (Andrews and Hammack, 2003) and then incubated at 42°C for 24 h. Following incubation, one loopful from each bottle was streaked for isolation onto MLIA and incubated at 37°C for 24 h. Suspect *Salmonella* colonies were confirmed as described under the MPN procedures. The prevalence procedure was employed to increase the minimum detectable level of *Salmonella* in a 25g sample.

2.3.5 MC, a_W, and pH Analysis

The remaining composite litter samples were used for MC, a_W, and pH analysis. Moisture content of the litter was measured by drying 2.5 g of sample overnight (~ 18 h) in a forced-air convection oven (Blue M, Atlanta, GA) at 105°C. Water activity of litter samples was measured using a Decagon Model CX-3 Water Activity System (Decagon Devices Inc., Pullman, WA) according to the manufacturer's instructions. To determine litter pH, 1 g of sample was combined with 10 ml of deionized water, mixed by vortexing, and allowed to stand for 1 minute (Pope and Cherry, 2000). Litter pH was then measured using a Corning 220 pH meter with a G-P Combo with RJ probe (Corning Incorporated, Corning, NY).

2.3.6 Statistical Analysis

All data were analyzed using the mixed procedure for analysis of variance (ANOVA) of SAS (SAS Institute Inc., 1996) according to the following model: $Y_{ijkl} = \mu + \alpha_i + \beta_j + C_{k(j)} + (\alpha\beta)_{ij} + (\alpha C)_{ik(j)} + E_{ijkl}$, where Y_{ijkl} was the observed dependent variable (fecal or litter *Salmonella* population); μ the overall mean; α_i the fixed effect of season (summer or winter); β_j the fixed effect of age (3 or 19 weeks of age); $C_{k(j)}$ the random effect of farm which is nested in the age effect (total of 12 farms); $(\alpha\beta)_{ij}$ the interaction between season and age;

$(\alpha C)_{ik(j)}$ the interaction between season and farm (nested in age); and E_{ijkl} is the random error. For statistical analysis farms served as experimental units and were considered a random effect nested in the age effect, unless otherwise stated. Variable means having a significant F-test ($P < 0.05$) were compared using the least-square-means (lsmeans) function of SAS and were considered to be significant at $P < 0.05$. All MPN data were transformed to its base-10 logarithm before analysis. Besides running the mixed procedure, the data were also analyzed using the regression and correlation procedures of SAS (indicated by R^2 and r values, respectively). MPN and prevalence results were examined using the Chi-Square frequency analysis procedure of SAS and variables were considered to be significant at $P < 0.05$.

2.4 RESULTS AND DISCUSSION

2.4.1 MPN Analysis and Intrinsic Characteristics of the Litter

The *Salmonella* populations detected on the commercial turkey farms are summarized in Tables 1 (litter samples) and 2 (fecal samples). All farms were *Salmonella*-positive at least one time throughout the study (minimum detection level = 1 log MPN/g). *Salmonella* population estimations ranged from <1 to ≥5.3 log MPN/g for litter samples and from <1 to 4.9 log MPN/g for fecal dropping samples. Only 1 farm was *Salmonella*-negative for both litter and fecal samples during the winter months (F9).

Age of the birds significantly influenced the litter *Salmonella* populations. Litter collected from farms housing the younger birds had significantly higher mean populations compared to older birds (4.08 vs. 2.07 log MPN/g, respectively; $P = 0.0017$, SEM = 0.3337).

Season and farm variables did not significantly influence *Salmonella* populations in the litter; however, there was significant *Salmonella* population variability in the fecal

samples due to season by farm interactions within age groups (season*farm(age) interaction, P = 0.0151). The mean *Salmonella* population of 3-week old turkeys reared during the winter months was 3.4 log MPN/g of feces in comparison to 1.9 log for 19-week old birds reared on the same season (1.5 log difference). In contrast, during the summer months both age groups averaged 2.7 log MPN/g of feces.

Using linear regression analysis, a significant interaction was observed between litter and fecal *Salmonella* populations (P = 0.0006, R^2 = 0.21), demonstrating that the litter *Salmonella* populations are significantly related to the fecal *Salmonella* populations. The positive relationship detected by these two sample types indicates that the analysis of the litter samples for *Salmonella* may be a good indicator of fecal *Salmonella* populations.

The intrinsic physical and chemical characteristics of the litter by season and age are summarized in Tables 3 and 4, respectively. As expected, litter temperatures were 6^{o}C higher during the summer, but did not differ according to bird age. Although ammonia levels are generally higher in the winter months as a result of reduced ventilation rates to conserve heat (Kristensen and Wathers, 2000), in this study ammonia emissions were 7.1 ppm higher during the summer months. This finding may be explained by other factors such as litter a_W, temperature, and pH (Table 3). As expected, ammonia levels were 12 ppm higher in older turkeys. These increases are related to the higher volume of fecal waste generated by older birds as well to higher litter a_W and moisture (Table 4). Moisture content and temperature of the litter were also positively correlated to ammonia levels (P = 0.0006, r = 0.48; P = 0.0389, r = 0.30; respectively). In a previous study (Pope and Cherry, 2000), litter pH levels were positively correlated to the concentration of NH_3 emissions from litter; therefore, as litter pH

increased there was an associated increase in NH_3 emission. However, in the present study, no significant correlation was found between litter pH and NH_3 levels.

The physical and chemical properties of litter monitored in this study did not significantly influence the litter *Salmonella* populations when they were used as covariates for the GLM analysis. However, a significant positive linear correlation was detected between litter *Salmonella* populations and pH ($r = 0.5$, $P = 0.0006$), whereas NH_3 and MC were negatively correlated to litter *Salmonella* populations ($r = -0.3$ and $r = -0.4$, $P = 0.02$ and $P = 0.009$, respectively). Pope and Cherry (2000) reported a significant reduction in aerobic bacterial populations of litter samples as litter pH dropped. Payne and coworkers (2002) also reported that *Salmonella* populations can be significantly reduced by acidifying litter with a litter amendment product. A similar response was observed in the present study where lower *Salmonella* populations were detected in more acidic litter (i.e., pH < 6 resulted in <2 log MPN/g).

As expected, litter moisture content (MC) and water activity (a_W) were positively correlated ($P < 0.0001$, $r = 0.60$). However, the regression analysis did not show any relationship between a_W and litter *Salmonella* populations ($P = 0.6049$, $R^2 = -0.01$) yet MC was significantly correlated to the *Salmonella* populations. Although some studies have shown that a low a_W environment, specifically lower than 0.84, can significantly reduce or limit growth of *Salmonella* (Himathongkham et al., 1999; Haynes et al., 2000), 10.4% of the litter *Salmonella* isolates in this study came from samples that had a_W lower than 0.84 and populations ranging from 1.4 to >4.7 log MPN/g. Lower moisture environments should in theory limit *Salmonella* populations, yet 44% of the *Salmonella* isolates came from samples with MC of <30%. Furthermore, the lowest litter *Salmonella* populations were recovered

from samples having the highest MC levels (Figure 1). These results agree with Williams (Willians, 1978) who concluded that *Salmonella* is poorly recovered from moist litter. Moreover, Christian and Scott (Christian and Scott, 1953) reported that although *Salmonella* populations increased significantly when exposed to a_W values from 0.93 to 0.95, some population reductions were observed at very high a_W values (> 0.99). This finding may also be related to the possibility that at increased MC, poultry litter may contain a higher population of native microorganisms capable of out-competing *Salmonella*. Also, a very moist litter may dilute nutrients or produce hypotonic conditions unfavorable for bacterial survival or multiplication.

2.4.2 Comparison between MPN and Prevalence Methods

Forty eight individual litter and fecal samples were collected from 12 turkey farms. The results of the frequency analysis for both litter and fecal samples (i.e., MPN procedure vs. prevalence method) are summarized in Tables 5 and 6.

Both methods were in agreement on 42 of 75 total *Salmonella*-positive samples (i.e., individual samples that were *Salmonella*-positive by both methods). For litter samples, both methods agreed on 26 of 38 total positive samples. The prevalence method yielded 9 of 48 (18.8%) false negatives (i.e., individual samples that were *Salmonella*-positive by one method but not the other) while the MPN method yielded only 3 of 48 (6.3%). However, no statistical differences were found between the two methods for the litter samples. For fecal samples, both methods agreed on 16 of 37 total positive samples. Moreover, the prevalence method generated 20 of 48 (41.7%) false negatives while the MPN method yielded only 1

false negative (2.1%) plus a significantly higher proportion of *Salmonella*-positive fecal samples (37.5%) ($\chi^2 = 15.2$, $P < .0001$).

The MPN enumeration method is a much more labor intensive and costly procedure than the prevalence method; however, it yields estimates of *Salmonella* populations instead of merely indications of presence or absence of the organism. Findings from this study demonstrated that the MPN procedure was significantly more sensitive than the prevalence procedure (for fecal samples). As previously suggested in several other studies, it would be reasonable to propose the use of BPW instead of lactose broth as a pre-enrichment broth for *Salmonella* prevalence assays (Moriñigo et al., 1986; Martin and McCann, 1998; Dufrenne et al., 2001).

In conclusion, the findings of this study indicate that *Salmonella* prevalence and populations in turkey litter and feces are significantly impacted by flock age and season by farm (within age) interactions. Rearing young birds on newer litter, especially when not using a litter treatment product, may lead to higher *Salmonella* litter populations in the brooder house. Furthermore, litter *Salmonella* populations were also influenced by litter pH, moisture content, and ammonia levels. In most cases, higher ammonia levels were associated with lower *Salmonella* populations. Moreover, a more acidic and wetter litter had lower *Salmonella* populations. Except for MC, season influenced all measured litter properties which were significantly lower during the winter. In addition, brooder farms had lower MC, a_W, and NH_3 levels compared to grow-out farms. This study successfully determined pre-harvest *Salmonella* populations thus providing important information that can aid in developing new and effective control strategies for reducing the level and incidence of *Salmonella* contamination of flocks entering processing plants.

2.5 CONCLUSIONS AND APPLICATIONS

1. *Salmonella* was present in 70.1% of the fecal and 79.2% of the litter samples taken during the brooder and grow-out phases of commercial turkey production.
2. *Salmonella* populations were approximately 2 logs/g higher in the litter samples from 3-week old turkeys compared to litter samples taken from houses containing 19-week old turkeys.
3. Season (summer vs. winter) did not influence *Salmonella* litter or fecal populations, yielding an overall average MPN population of 3.0 log/g of sample.
4. Because of financial and labor constraints, the total number of samples analyzed was limited; therefore, deriving relationships between the measured litter characteristics and *Salmonella* populations will require further study involving a larger number of samples.
5. These results provide evidence that *Salmonella* can be present at high populations during turkey production, even when bacterial cells are exposed to apparent adverse environments. These findings suggest that intervention strategies should aggressively target critical points such as better litter management on brooder farms and reduced temperature-induced stresses.

2.6 TABLE AND FIGURES

Table 1. Log most probable number (MPN)[1] of *Salmonella* per gram of turkey litter samples

	FARM													
	3 WEEKS							19 WEEKS						
SEASON	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**Avg[5]**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**Avg**
Winter[2]	1.4	3.9	2.5	>4.7[4]	>5.2	>5.2	3.8	2.5	2.5	<1.0[6]	1.8	2.5	2.5	2.1
Summer[3]	4.8	1.8	4.6	5.3	4.3	>5.3	4.3	2.9	2.7	2.4	1.2	<1.0	1.5	2.0

Statistical Analysis

Source	**P-value**
Season	0.7591
Age	0.0017[7]
Season x Age	0.4825
Farm(Age)	0.4757
Season x Farm(Age)	0.0987

[1]Base-10 logarithm of the most probable number of *Salmonella* present per gram of litter sample (average of 2 turkey houses within a farm).

[2]Samples taken between November and March.

[3]Samples taken between April and October.

[4]>: all MPN tubes were positive.

[5]Avg: average means.

[6]<: negative results from both microbiological methods: reported as below the detection limit of MPN procedure: 10 cells/gram of sample (1 log MPN/g).

[7]SEM(10): standard error of the mean with 10 degrees of freedom = 0.3337.

Table 2. Log most probable number (MPN)[1] of *Salmonella* per gram of turkey fecal samples

	FARM													
	3 WEEKS							19 WEEKS						
SEASON	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**Avg[5]**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**Avg**
Winter[2]	2.2	>4.1[4]	<1.0[5]	>4.7	3.6	4.6	3.4	2.7	2.8	<1.0	1.5	1.8	1.4	1.9
Summer[3]	3.3	1.0	2.6	2.8	1.3	4.9	2.7	2.5	2.3	4.2	4.3	1.1	1.7	2.7

Statistical Analysis

Source	**P-value**
Season	0.9211
Age	0.1841
Season x Age	0.1789
Farm(Age)	0.5800
Season x Farm(Age)	0.0151[7]

[1]Base-10 logarithm of the most probable number of *Salmonella* present per gram of fecal sample (average of 2 turkey houses within a farm).

[2]Samples taken between November and March.

[3]Samples taken between April and October.

[4]>: all MPN tubes were positive.

[5]<: negative results from both microbiological methods: reported as below of the detection limit of MPN procedure: 10 cells/gram of sample (1 log MPN/g).

[6]Avg: average means.

[7]Covariate parameter estimate: 1.1648 (Residual estimate = 1.1648).

Table 3. Intrinsic properties of litter samples of turkeys during winter and summer[1]

Measurements[2]	Winter	Summer	P-value	SEM
pH	6.59^{b}	7.28^{a}	0.0002	0.1170
a_W	0.883^{b}	0.942^{a}	0.0135	0.0160
MC (%)	29.7	29.3	0.8412	1.1400
Temperature (ºC)	22.3^{b}	28.3^{a}	<.0001	0.4218
NH_3 (ppm)	3.8^{b}	10.9^{a}	0.0014	1.4401

[1]Average of all farms within seasons but across ages (3 and 19 wk-old).

[2]a_w – water activity, MC – moisture content.

[a,b] Means with different superscripts within a row differ significantly ($P < 0.05$).

Table 4. Intrinsic properties of litter samples of 3 and 19 week-old turkeys[1]

Measurements[2]	3-week	19-week	P-value	SEM
pH	7.09	6.79	0.0833	0.1170
a_W	0.879^b	0.945^a	0.0064	0.0160
MC (%)	21.2^b	37.8^a	<.0001	1.1400
Temperature (ºC)	25.7	24.9	0.1953	0.4218
NH_3 (ppm)	1.3^b	13.3^a	<.0001	1.4401

[1]Average of all farms within ages but across seasons (winter and summer).

[2]a_w – water activity, MC – moisture content.

[a,b] Means with different superscripts within a row differ significantly ($P < 0.05$).

Table 5. Frequency analysis of two *Salmonella* detection methods from turkey litter samples[1]

Method	*Salmonella*-Positive Samples (%)	*Salmonella*-Negative Samples (%)	Total (%)
MPN[2]	35 (36.5)	13 (13.5)	48 (50.0)
Prevalence	29 (30.2)	19 (19.8)	48 (50.0)
Total (%)	64 (66.7)	32 (33.3)	96 (100.0)

[1]$\chi^2 = 1.7$, DF = 1, P = 0.1939.

[2]MPN – most probable number enumeration method.

Table 6. Frequency analysis of two *Salmonella* detection methods from turkey fecal samples[1]

Method	*Salmonella*-Positive Samples (%)	*Salmonella*-Negative Samples (%)	Total (%)
MPN[2]	36 (37.5)	12 (12.5)	48 (50.0)
Prevalence	17 (17.7)	31 (32.3)	48 (50.0)
Total (%)	53 (55.2)	43 (44.8)	96 (100.0)

[1] $\chi^2 = 15.2$, DF = 1, $P < 0.0001$.

[2] MPN – most probable number enumeration method.

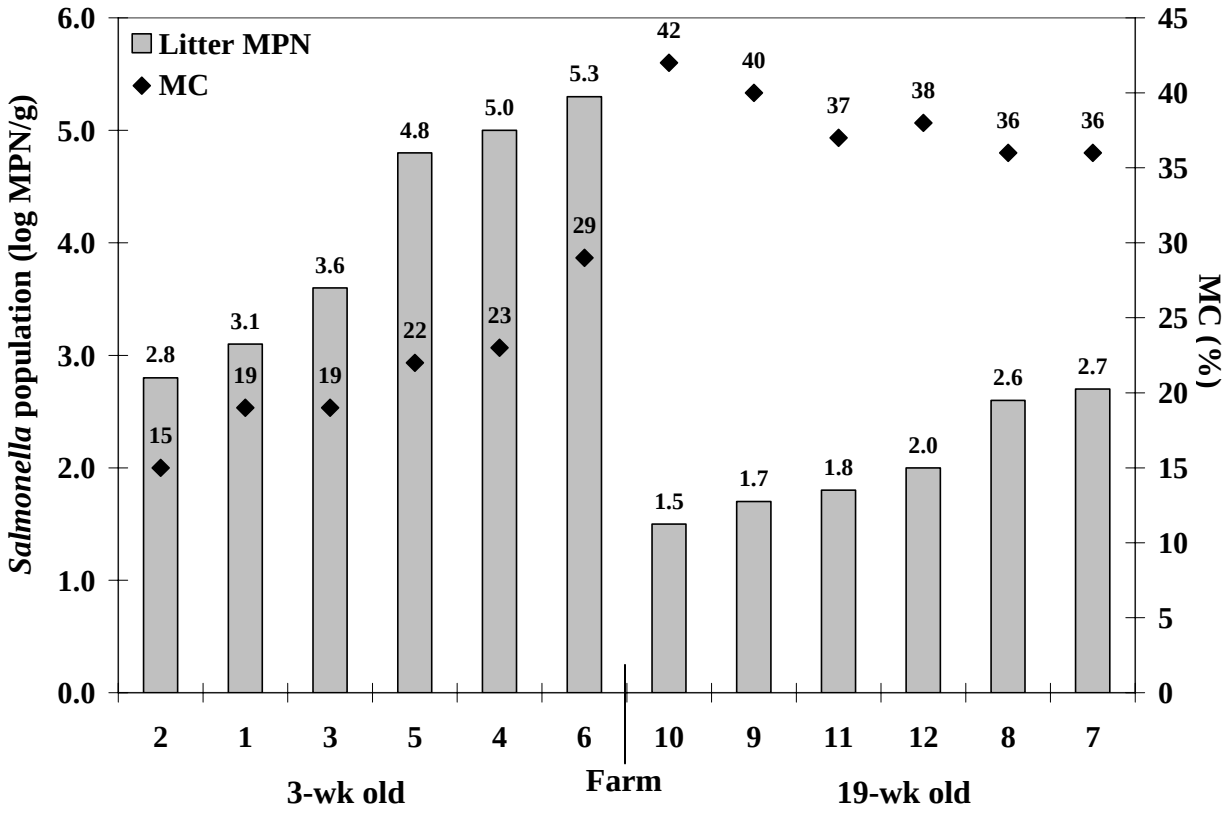

Figure 1. Litter moisture content (%) and *Salmonella* populations (log MPN/g) as influenced by farm and age (across seasons)

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CHAPTER 3

GENOTYPES, SEROTYPES AND ANTIBIOTIC RESISTANCE PROFILES OF *SALMONELLA* ISOLATED FROM COMMERCIAL NORTH CAROLINA TURKEY FARMS[*]

[*] Use of trade names in this publication does not imply endorsement by the North Carolina Agriculture Research Service or the North Carolina Cooperative Extension Service of the products mentioned, nor criticism of similar products not mentioned.

3.1 ABSTRACT

Bacterial typing methods such as serotyping, pulsed-field gel electrophoresis (PFGE), and antibiotic resistance patterns (AbR) have been used in epidemiological investigations to elucidate the origin and mode of transmission of pathogens. Once the route of transmission is identified, control measures are implemented to avoid future outbreaks. The objective of this study was to determine the serotypes, genotypes and AbR patterns of 42 *Salmonella enterica* subsp. *enterica* isolates recovered from either fecal or litter samples of 12 commercial turkey farms across two seasons (summer, winter) and two ages (3, 19 wk). Isolates were serotyped based on the Kauffmann-White Scheme. Genotyping was done by restriction digestion of chromosomal DNA (*Xba*I) and subsequent PFGE, and AbR was determined using Sensititre susceptibility plates. Serovar Kentucky was the most prevalent serotype (26%), followed by Senftenberg (19%), Muenster (17%), Mbandaka (10%), Javiana (7%), Hadar (5%), Heidelberg (5%), 8,(20):nonmotile (5%), Agona (2%), Infantis (2%) and 4,12:r:- (2%). Serovars Kentucky, Heidelberg, Hadar and 8,(20):nonmotile were only isolated from the 19-wk bird samples, whereas Senftenberg and Muenster were only isolated from young birds (3-wk). Isolates within any one serotype showed minor PFGE banding pattern differences, but dendogram analysis indicated that sequence variability between serotypes was more significant than within serotypes. Isolates were resistant to tetracycline (86%), sulfisoxazole (71%), streptomycin (64%), gentamicin (41%), ampicillin (36%), kanamycin (26%), sulfamethoxazole trimethoprim (7%), nalidixic acid (5%), cefoxitin (2%) and ceftiofur (2%). One isolate (Muenster) was resistant to 9 antibiotics (2%), the others were resistant to 6 (17%), 5 (12%), 4 (10%), 3 (21%), 2 (24%) and 1 (10%) antibiotic. Only 2 isolates

(5%) were susceptible to all antibiotics tested. AbR patterns were affected by age; on average strains recovered from young birds were resistant to >4 drugs compared to <3 in older birds ($P<0.05$). This study showed that *Salmonella enterica* subsp. *enterica* serotypes, genotypes and AbR patterns were affected by bird age but not by season or farm.

Keywords: *Salmonella*, serotype, turkeys, farm, PFGE, antibiotic resistance

3.2 INTRODUCTION

Salmonella was first isolated in the United States in 1885 by Salmon and Smith from swine suffering from hog cholera and was named *Bacillus cholerae-suis* (Le Minor, 1981). However, this genus was the cause of serious disease long before its first isolation. In the early 19th century, the connection between human intestinal ulceration and a contagious agent, later identified as typhoid fever, was first documented by French pathologists (D'Aoust, 2001). The generic term *Salmonella* was first used by Lignières in 1900 after the development of improved serological techniques that enabled the identification of somatic (O) and flagellar (H) antigens (Le Minor, 1981). In 1926, the first antigenic scheme for the classification of salmonellae was proposed by White, which was expanded by Kauffmann in 1941 to subsequently become the Kauffmann-White Scheme (D'Aoust, 2001).

Since the development of the first antigenic scheme for *Salmonella* classification, more than 2500 serovars have been identified (Popoff et al., 2004). Recently, several alternative typing methods have been used in epidemiological investigations intended to

elucidate the origin and mode of transmission of *Salmonella*. Some examples are pulsed-field gel electrophoresis (PFGE), ribotyping, antibiotic resistance patterns (AbR), plasmid profiling, multilocus sequence typing (MLST), and random amplification of polymorphic DNA (RAPD). Nonetheless, serotyping remains an important identification tool used to investigate *Salmonella* epidemiology. For the purpose of this document serotypes of *Salmonella enterica* subsp. *enterica* will be referred as *Salmonella* accompanied by the serovar name, for example *Salmonella enterica* subsp. *enterica* serovar Typhimurium will be referred as *Salmonella* Typhimurium (or *S.* Typhimurium). The Public Health Laboratory Information System (PHLIS) surveillance data has shown that the most common *Salmonella* serotypes isolated from turkey sources in 2003 were *Salmonella* Senftenberg, Hadar and Heidelberg (CDC, 2004). Additionally, a recent study detected both horizontal and vertical transmission of two *Salmonella* serotypes, Typhimurium and Enteritidis, in a commercial broiler company (Lijabjelke et al., 2005). The investigators were able to show that *S.* Typhimurium and Enteritidis isolates from breeder flocks, chick-box liners (hatchery), the broiler flock environment and carcass rinses of processed broilers had identical PFGE patterns, suggesting both vertical and horizontal transmission of these serotypes. Unfortunately, serotyping is not always discriminatory enough to identify the source of a common *Salmonella* serotype, it is generally recognized that a single method cannot be relied upon for discriminating between strains in studying the ecology of *Salmonella*.

Pulsed-field gel electrophoresis is a very discriminatory and reproducible typing method which is commonly used in epidemiological investigations. Previous studies have shown that PFGE provides valuable information that can be used to differentiate

Salmonella serotypes; in fact, the method allows detection of DNA polymorphisms that were previously undetected by other techniques, such as ribotyping (Liebana et al., 2002; Nayak et al., 2004). For example, Nayak et al. (2004) suggested that PFGE had superior discriminatory power as compared to ribotyping when used to classify *Salmonella* serotypes isolated from turkey samples. Moreover, Cardinale et al. (2005) have shown that *Salmonella* isolates from humans and poultry share similar PFGE profiles. However, the relationship between *Salmonella* serotypes and genotypes at the farm level remains unknown, as well as their movement within the turkey production phases.

Antimicrobial resistance profiling has also been used to investigate *Salmonella* ecology at the farm level (Nayak et al., 2004). Increasing antimicrobial resistance among *Salmonella* has been noted for several decades (Lee et al., 1994), particularly among strains of the serotype Typhimurium (Rabatsky-Ehr et al., 2004). Nayak et al. (2004) showed that the combination of PFGE, serotyping and antibiotic resistance profiling could better explain the transmission of antibiotic-resistant *Salmonella* within a turkey production facility.

Although considerable studies have been conducted to understand the molecular diversity of *Salmonella* in broiler chickens, limited research and information is available for the turkey industry. In reality, turkey production is considerably longer than broiler production and management requirements of turkeys are different than those of broilers. These differences may result in different *Salmonella* behavior and ecology. Based on the hypothesis that *Salmonella* strain profiles are influenced by season, farm and turkey age, the objective of this study was to determine the serotypes, genotypes and antibiotic resistance/susceptibility patterns of 42 *Salmonella* isolates recovered from either fecal or

litter samples of 12 commercial turkey farms across two seasons (summer and winter) and two turkey ages (3 and 19 weeks).

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains

Forty-two *Salmonella* isolates recovered from fecal and litter samples taken from two houses per farm across twelve commercial turkey farms and two seasons, winter (November through March) and summer (April through October), were randomly chosen for further characterization. All isolates were collected in a previous study between the years 2002 and 2003 (Santos et al., 2005). Briefly, each turkey farm consisted of curtain-sided ventilated houses, clay-lined floors, with pine shavings serving as bedding material. All brooder and grow-out farms followed similar management practices since they were contracted by the same parent integrator company. Moreover, litter was removed and houses cleaned and sanitized between flocks. The farms were coded as F1 through F6 for 3 week-old birds (brooder farms) and F7 through F12 for 19 week-old birds (grow-out farms). Because of logistical and integrator policies, no attempt was made to follow the same flock from the brooder phase through the grow-out phase of production.

3.3.2 Selection of Isolates

Twenty-one *Salmonella* isolates were selected from both litter and fecal samples and were representative of each age, farm and season. Isolates were confirmed biochemically by inoculating an isolated colony onto triple sugar iron (TSI) agar slants (Oxoid Ltd., Ogdensburg, NY) and then incubating the slants at 37°C for 24 h, as well as

serologically by agglutination using poly-O antiserum (Difco, Sparks, MD). From each confirmed positive sample, one well-isolated colony was picked and re-streaked onto modified lysine iron agar (MLIA, Oxoid Ltd., Ogdensburg, NY) plates and incubated at 37°C for 24 h. After incubation, isolates were re-tested using poly-O antiserum agglutination kit (Difco, Sparks, MD) and subsequently cultured in brain heart infusion (BHI) broth (Oxoid Ltd., Ogdensburg, NY) at 37°C for 18 h. One milliliter of each BHI tube was mixed with 1 ml of freezing solution [4× BHI broth plus 40% glycerol (Fisher Scientific International, Bohemia, NY)] and frozen in a -20°C freezer for further analysis.

3.3.4 Serotyping

Frozen cultures were resuscitated in BHI broth (37°C for 24 h), transferred to MLIA agar and incubated at 37°C for 24 h. One isolated colony was randomly picked from each sample plate, streaked onto tryptic soy agar (Difco, Sparks, MD) and shipped to the National Veterinary Service Laboratories, Animal and Plant Health Inspection Services – United States Department of Agriculture, Ames, IA (NVSL, APHIS–USDA) for serotype determination. Serotyping was done based on the Kauffmann-White Scheme.

3.3.5 Antibiotic Resistance/Susceptibility Profiling

The antibiograms and partial range minimum inhibitory concentration (MIC) were determined for the same 42 serotyped isolates as described earlier (CDC, 2002) using the Sensititre NARMS gram-negative susceptibility plates (Trek Diagnostic Systems, Cleveland, OH) containing the following 15 antimicrobial agents (acronyms and dilution ranges are indicated in parenthesis): amikacin (A, 0.5-64 μg/ml),

amoxicillin/clavulanic acid (Ax/C, 1-32/0.5-16 μg/ml), ampicillin (Ap, 1-32 μg/ml), cefoxitin (C, 0.5-32 μg/ml), ceftiofur (Ct, 0.12-8 μg/ml), ceftriaxone (Cx, 0.25-64 μg/ml), chloramphenicol (Ch, 2-32 μg/ml), ciprofloxacin (Cp, 0.015-4 μg/ml), gentamicin (G, 0.25-16 μg/ml), kanamycin (K, 8-64 μg/ml), nalidixic acid (N, 0.5-32), streptomycin (St, 32-64 μg/ml), sulfisoxazole (Sx, 16-256 μg/ml), tetracycline (T, 4-32 μg/ml), and trimethoprim/sulfamethoxazole (Tr/S, 0.12-4/2.38-76 μg/ml). Isolates were streaked onto MLIA and grown overnight at 37°C. Following incubation, 2 isolated colonies were collected and transferred to 5 ml of sterile 0.9% saline solution and adjusted to a 0.5 McFarland (ca. 1 x 10^8 CFU/ml, Fisher Scientific International, Bohemia, NY) using a nephelomenter (Trek Diagnostic Systems, Cleveland, OH). Seventy-five microliters of saline suspension were then transferred to 10 ml of Hinton-Mueller (MH) broth (Oxoid Ltd., Ogdensburg, NY) and 50 μl of inoculated MH broth were transferred to each plate well. Plates were sealed and incubated at 37°C for 16-20 hours. After incubation, plates were manually read using a microtiter plate holder (Trek Diagnostic Systems, Cleveland, OH). The National Committee for Clinical Laboratory Standards (NCCLS) MIC breakpoint standards (NCCLS, 2002) were used for determining antimicrobial resistance, with the exception of ceftiofur and streptomycin. *Salmonella* isolates were considered resistant whenever growth was observed at the following concentrations: ≥ 32 μg/ml for amikacin, ampicillin, chloramphenicol, cefoxitin, and nalidixic acid; ≥ 32/16 μg/ml for amoxicillin/clavulanic acid; ≥ 64 μg/ml for ceftriaxone; ≥ 4 μg/ml for ciprofloxacin; ≥ 8/152 μg/ml for trimethoprim/ sulfamethoxazole; ≥ 8 μg/ml for gentamicin; ≥ 25 μg/ml for kanamycin; ≥ 350 μg/ml for sulfisoxazole; and ≥ 16 μg/ml for tetracycline.

Resistance to ceftiofur and streptomycin was defined as a MIC of ≥ 8 μg/ml and ≥ 64 μg/ml, respectively, as previously described (Rabatsky-Ehr et al., 2004).

3.3.6 DNA Extraction/Purification and Pulsed-Field Gel Electrophoresis Procedure

DNA preparation and PFGE procedures were followed as described previously (Liebana et al., 2002) with some modifications. Single colonies of *Salmonella* isolates were incubated overnight at 37ºC in 10 ml of Luria-Bertani (LB) broth (Oxoid Ltd., Ogdensburg, NY) with moderate shaking (120 rpm). One milliliter aliquots of the cultures were pelleted and washed twice with 1 ml phosphate-buffered saline (PBS) (Fisher Scientific International, Bohemia, NY), the cells re-suspended in 0.8 ml of PBS and then equilibrated at 40ºC. This suspension was mixed in equal parts with molten 2% agarose (Bio-Rad, Hercules, CA) and pipetted into disposable molds (Bio-Rad, Hercules, CA). Plugs were incubated overnight at 56ºC in 2 ml of lysis buffer (0.5 M EDTA (Promega, Madison, WI), 1% N-laurylsarcosine (Fisher Scientific International, Bohemia, NY) with proteinase K (Qiagen Inc., Valencia, Ca) at a final concentration of 250 mg/ml. After incubation, DNA-containing plugs were thoroughly washed in TE buffer [10 mM Tris–HCl (Bio-Rad, Hercules, CA), 1 mM EDTA, pH 8] and digested overnight with 30 U of *Xba*I restriction enzyme (Fisher Scientific International, Bohemia, NY). PFGE was performed with a CHEF DRII system (Bio-Rad, Hercules, CA) in 0.5× TBE buffer (130 mM Tris, 45 mM boric acid, 2.5 mM EDTA, Fisher Scientific International, Bohemia, NY) with recirculation at 14ºC. DNA macrorestriction fragments were resolved on a 1% agarose gel with a Lambda (λ) ladder PFG marker (New England Biolabs, Ipswich, MA) used as a size standard. Pulse times ramped from 6 to 40 s during

a 20 h run at 6 V/cm. PFGE macrorestriction patterns were compared using Bio-Numerics Software (BioNumerics version 3.5, Applied Maths BVBA, Austin, TX).

3.3.7 Statistical Analysis

Serotype frequency was analyzed using the frequency analysis of SAS (Chi-Square) and results were considered to be significant at $P < 0.05$ (SAS, 1996). Antibiotic resistance patterns were analyzed as a 12×2×2 factorial design and means were separated using the mixed procedure for analysis of variance (ANOVA) of SAS according to the following model: $Y_{ijkl} = \mu + \alpha_i + \beta_j + C_{k(j)} + (\alpha\beta)_{ij} + (\alpha C)_{ik(j)} + E_{ijkl}$, where Y_{ijkl} was the observed dependent variable (number of antibiotics an isolate was resistant to); μ the overall mean; α_i the fixed effect of season (summer or winter); β_j the fixed effect of age (3 or 19 weeks of age); $C_{k(j)}$ the random effect of farm which is nested in the age effect (total of 12 farms); $(\alpha\beta)_{ij}$ the interaction between season and age; $(\alpha C)_{ik(j)}$ the interaction between season and farm (nested in age); and E_{ijkl} is the random error. For statistical analysis farms served as experimental units and were considered a random effect nested in the age effect, unless otherwise stated. Variable means having a significant F-test ($P < 0.05$) were compared using the least-square-means (lsmeans) function of SAS and were considered to be significant at $P < 0.05$. PFGE macrorestriction patterns were compared using Bio-Numerics Software. Cluster analyses using the dice coefficient for band matching with a 1% position tolerance and the single linkage method were used to generate the dendogram describing the relationship among *Salmonella* genotypes.

3.4 RESULTS AND DISCUSSION

3.4.1 Serotyping

The *Salmonella* serotypes identified are summarized in Table 1. All serotypes were members of the species *Salmonella enterica* subspecies *enterica*. Serovar Kentucky was the most prevalent serotype (26%) followed by *S.* Senftenberg (19%) and *S.* Muenster (17%). The *Salmonella* serotypes identified in this study have been previously identified as common turkey serotypes (Byrd et al., 1997; Roy et al., 2002). In the 2003 *Salmonella* Annual Summary Report, the 5 most frequently isolated serotypes from non-clinical turkey samples were: Senftenberg, Hadar, Heidelberg, Muenster and Kentucky (CDC, 2004). Previous studies have indicated that *S.* Kentucky is one of the most frequently isolated serotypes from poultry, poultry products, and the poultry production environment (Byrd et al., 1997; Roy et al., 2002). Although *S.* Heidelberg is commonly isolated from turkey samples and the turkey farm environment (Nayak et al., 2004), the frequency of isolating *S.* Heidelberg in the present study was relatively low (4.8%). Statistical analysis of serotype frequency revealed a significant age effect such that serotypes Kentucky, Heidelberg, Hadar and 8,(20):nonmotile were isolated only from samples obtained from 19-week old turkeys whereas Senftenberg and Muenster were only isolated from 3-week old poults ($\chi^2 = 36.3$, $P < 0.001$, Figure 1). Type of sample (fecal vs. litter) and season (winter vs. summer) did not significantly influence serotype frequency ($P > 0.05$). Based on the findings reported in Table 2, it is apparent that *Salmonella* positive samples taken from both fecal and litter samples contained organisms of the same serotype, suggesting that the serotypes recovered from the litter were shed by the host. For example, both isolates of the Hadar serovar came from the

same farm, same season but different sources; one was recovered from fecal samples and the other from litter. Previous studies have shown that the cycling of *Salmonella* between contaminated litter and the bird's gastrointestinal tract is a significant factor for maintaining intestinal infection in flocks (Jones et al., 1991; Corrier et al., 1999).

3.4.2 Antibiotic Resistance/Susceptibility Profiling

The antimicrobial agents to which *Salmonella* isolates (n = 42) were most commonly resistant to were tetracycline (85.7%), sulfisoxazole (71.4%), streptomycin (64.3%), gentamicin (40.5%), ampicillin (35.7%), kanamycin (26.2%), trimethoprim/ sulfamethoxazole (7.1%), nalidixic acid (4.8%), cefoxitin (2.4%) and ceftiofur (2.4%). No *Salmonella* isolates were resistant to amikacin, amoxicillin/clavulanic acid, chloramphenicol, ceftriaxone and ciprofloxacin. Similar results were reported by Nayak and co-workers (2004), who found that *Salmonella* isolates obtained from turkey ceca and the farm environment were more often resistant to streptomycin (62%), gentamicin (52%), tetracycline (31%) and trimethoprim/sulfamethoxazole (3%). In the same study, no isolates were resistant to chloramphenicol, ciprofloxacin and ceftriaxone. This high degree of antibiotic resistance was further reflected in the MIC values for tetracycline (> 32 μg/ml), sulfisoxazole (> 256 μg/ml), streptomycin (> 64 μg/ml), gentamicin (> 16 μg/ml), ampicillin (> 32 μg/ml) and kanamycin (> 64 μg/ml). The elevated resistance to gentamicin may be explained by the fact that most turkey breeders dip hatching turkey eggs in gentamicin sulfate solutions to prevent mycoplasmosis (Hirsch et al., 1983). Additionally, gentamicin has also been used to prevent *Escherichia coli* infection in day-old poults as well as to treat poults and turkeys infected with *Salmonella enterica* subsp.

arizonae (Poppe et al., 1995, Nayak et al., 2004). The degree of resistance to tetracycline observed in our study was much higher than that reported by Poppe et al. (38.1%) (1995) and Nayak et al. (31%) (2004).

There is considerable concern among the medical community and regulatory agencies over the increasing resistance of bacterial pathogens to fluoroquinolones such as ciprofloxacin, which are also used for the treatment of human foodborne infections (Asperilla et al., 1990; Angulo et al., 2000). In the present study, none of the *Salmonella* isolates were resistant to ciprofloxacin. Although the *Salmonella* isolates were sensitive to ciprofloxacin, two isolates (4.8%) were resistant to the quinolone nalidixic acid. The resistance of *Salmonella* isolates to quinolones and fluoroquinolones has been previously reported (Angulo et al., 2000; CDC, 2004).

Multiple-drug resistant *Salmonella* serotypes have been isolated from farm animals and foods of animal origin (Nayak et al., 2004, Rabatsky-Ehr et al., 2004). In the present study, several *Salmonella* serotypes exhibited resistance to multiple antimicrobial agents. For example, one of the *S.* Muenster isolates was resistant to 9 of the 15 antibiotics tested (Table 3). Other serotypes were resistant to 6 (17%, Muenster, Senftenberg), 5 (12%, Javiana, Mbandaku, Muenster, Senftenberg), 4 (10%, Kentucky, Senftenberg), 3 (21%, 7 serotypes), 2 (24%, Kentucky, 4,12:r:-) and 1 (10%, Heidelberg, Kentucky) antibiotic. Only 2 *Salmonella* isolates (5%, Infantis, Senftenberg) were susceptible to all the antibiotics tested. Nayak and co-workers (2004) found similar multidrug-resistant isolates such as *S.* Muenster, which in that case was resistant to 7 antibiotics. Turkey age significantly influenced multiple-antibiotic resistance. In general, isolates from young birds (3-week old poults) were resistant to more than 4 drugs and

those from older birds (19-week old turkeys) were resistant to 3 or less antibiotics tested (P = 0.0273, Figure 2). Type of sample (fecal vs. litter) and season (winter vs. summer) did not significantly influence multiple-drug resistance of *Salmonella* isolates (P > 0.05).

3.4.2 Pulsed-Field Gel Electrophoresis Analysis

The PFGE of *Xba*I-digested chromosomal DNA from the 42 isolates gave stable and reproducible patterns consisting of 9-17 fragments (*Mean* = 14). Isolates within any one serotype showed minor PFGE banding pattern differences, but dendogram analysis indicated that sequence variability between serotypes was more significant than within serotypes. The genetic relatedness of the PFGE profiles, as demonstrated by the dendogram, showed a total of 12 PFGE groups or clusters (A – L) of 65% or greater relatedness (Figure 3). Most serotypes within each cluster had little band pattern differences and were grouped together; the exceptions were *S.* Senftenberg, Muenster and Javiana (Figure 3). Such genetic homogeneity among *Salmonella* serotypes was also documented by Nayak and co-workers (2004), who, after typing 29 *Salmonella* isolates, found only 5 PGFE clusters. Another study showed even more uniformity between isolates, where only 2 clusters were identified with an overall similarity of 92% (Cardinale et al., 2005). Turkey age also influenced genotype distribution (Figure 3). Genotypes from young turkey poults (3-weeks old) belonged in groups F, H, I, J, K, and L. In contrast, genotypes from older turkeys (19-weeks old) belonged to groups B, C, D, and G. Although isolates in groups A and E were represented by mixed ages, a higher proportion of the isolates were recovered from samples of 3-week old poults.

The use of molecular typing methods in combination with phenotypic methods facilitate the demonstration of some important characteristics of *Salmonella* movement and transmission within the turkey production sector. There was an apparent association between turkey age and genotype, serotype and antibiotic resistance profile. For instance, some serotypes were only present and shed by birds housed in brooder farms, where turkey poults are reared until they reach 6 weeks of age. Once birds were transferred to the grow-out farms, the pattern changed and different genotypes/serotypes appeared. A similar situation occurred with the antibiogram patterns. Multiple-antibiotic resistance was more frequently found in isolates recovered from turkey poult samples. This finding may be related to the more intensive use of antimicrobial drugs by the turkey industry during hatching and the early stages of turkey development to treat and/or prevent certain diseases such as mycoplasmosis, colibacillosis and arizoonosis (Hirsch et al., 1983; Poppe et al., 1995; Nayak et al., 2004). These findings are in agreement with our previous study (Santos et al., 2005) where *Salmonella* populations present in litter samples were significantly influenced by turkey age. For example, *Salmonella* populations were approximately 2 $\log_{10}$/g higher in the litter samples taken from 3-week old turkeys compared to litter samples taken from houses containing 19-week old turkeys.

3.5 CONCLUSIONS

1. *Salmonella* serotype distribution varied significantly depending on turkey age (χ^2 = 36.3, $P < 0.0001$). Serotypes Kentucky, Heidelberg, Hadar and 8,(20):nonmotile were only isolated from samples obtained from 19-week old turkeys, whereas Senftenberg

and Muenster were only isolated from 3-week old poults. Given the fraction of isolates that were analyzed, no farm or seasonal effects were observed.

2. Antibiotic resistance patterns of *Salmonella* serotypes were significantly influenced by turkey age (P = 0.027). Isolates from 3-week old poults were resistant to more than 4 drugs and those from 19-week old turkeys were resistant to 3 or less antibiotics. Again, within the confines of this study, neither farm nor seasonal effects were observed.
3. A total of 12 PFGE clusters of 65% or greater relatedness were observed across the 42 *Salmonella* isolates. Turkey age was associated with the distribution of genotypes between clusters.
4. The combined analysis of genotypic and phenotypic characteristics provides a more complete understanding of *Salmonella* movement during turkey production and confirms the significant influence of turkey age on isolate distribution.

ACKNOWLEDGMENTS

This study was supported by the North Carolina Agricultural Foundation and by the USDA Initiative for Future Agriculture and Food Systems (IFAFS) grant. The authors would like to acknowledge Dr. Xin Li, Dr. Anael Santos and Dr. Joshua Payne for their assistance. Appreciation is also extended to Dr. Sophia Kathariou and Robin Siletzky from Food Science Department, NCSU, Raleigh, NC for technical assistance and equipment support with the BioNumerics software.

3.6 TABLES AND FIGURES

Table 1. Frequency of *Salmonella* serotypes isolated from turkey samples (feces and litter)

SEROTYPE	NUMBER OF ISOLATES	%
S. Kentucky	11	26.2
S. Senftenberg	8	19.0
S. Muenster	7	16.7
S. Mbandaka	4	9.5
S. Javiana	3	7.1
S. 8,(20):nonmotile	2	4.8
S. Hadar	2	4.8
S. Heidelberg	2	4.8
S. 4,12:r:-	1	2.4
S. Agona	1	2.4
S. Infantis	1	2.4
Total	**42**	**100.0**

Table 2. Frequency of *Salmonella* serotypes identified in turkey litter and fecal dropping samples

SEROTYPE	% FECAL ISOLATES (n)[1]	% LITTER ISOLATES (n)[1]
S. Kentucky	14.3 (6)	11.9 (5)
S. Senftenberg	7.1 (3)	11.9 (5)
S. Muenster	9.5 (4)	7.1 (3)
S. Mbandaka	4.8 (2)	4.8 (2)
S. Javiana	2.4 (1)	4.8 (2)
S. 8,(20):nonmotile	2.4 (1)	2.4 (1)
S. Hadar	2.4 (1)	2.4 (1)
S. Heidelberg	2.4 (1)	2.4 (1)
S. 4,12:r:-	2.4 (1)	0.0 (0)
S. Agona	2.4 (1)	0.0 (0)
S. Infantis	0.0 (0)	2.4 (1)
Total	**50.0 (21)**	**50.0 (21)**

[1] Percentage of total *Salmonella* isolates serotyped.

Table 3. Antibiotic resistance profiles of *Salmonella* isolates[1] recovered from turkey fecal or litter samples

SEROTYPE	SOURCE	ANTIBIOTIC RESISTANCE PHENOTYPE
S. Agona	Fecal	St, Sx, T
S. Hadar	Fecal	St, Sx, T
S. Hadar	Litter	St, Sx, T
S. Heidelberg	Fecal	T
S. Heidelberg	Litter	T
S. Infantis	Fecal	*Susceptible to all antibiotics tested*
S. Javiana	Fecal	Ap, Sx, T
S. Javiana	Litter	Ap, G, St, Sx, T
S. Javiana	Litter	Ap, Sx, T
S. Kentucky	Fecal	St, T
S. Kentucky	Fecal	St, T
S. Kentucky	Fecal	T
S. Kentucky	Fecal	St, T
S. Kentucky	Fecal	St, Sx, T
S. Kentucky	Fecal	St, Sx, T
S. Kentucky	Litter	T
S. Kentucky	Litter	St, T
S. Kentucky	Litter	St, T
S. Kentucky	Litter	St, Sx, T
S. Kentucky	Litter	G, St, Sx, T
S. Mbandaka	Fecal	St, Sx, T
S. Mbandaka	Fecal	Sx, Tr/S
S. Mbandaka	Litter	G, N, Sx, St, Tr/S
S. Mbandaka	Litter	St, Sx, T
S. Muenster	Fecal	Ap, G, K, St, Sx, T
S. Muenster	Fecal	Ap, G, K, St, T
S. Muenster	Fecal	Ap, G, K, St, Sx, T
S. Muenster	Fecal	G,St, Sx
S. Muenster	Litter	Ap, G, K, St, Sx, T
S. Muenster	Litter	Ap, C, Ct, G, K, N, St, Sx, T
S. Muenster	Litter	Ap, G, K, St, T
S. Senftenberg	Fecal	Ap, G, K, St, Sx
S. Senftenberg	Fecal	Ap, St, Sx, T
S. Senftenberg	Fecal	Ap, G, K, St, Sx, T
S. Senftenberg	Litter	Ap, G, K, St, Sx
S. Senftenberg	Litter	G, St, Sx, T
S. Senftenberg	Litter	Ap, G, K, St, Sx, T
S. Senftenberg	Litter	Ap, G, K, St, Sx, T
S. Senftenberg	Litter	*Susceptible to all antibiotics tested*
S. 4,12:r:-	Litter	Sx, T
S. 8,(20):nonmotile	Fecal	Sx, T, Tr/S
S. 8,(20):nonmotile	Litter	Sx, St, T

[1] n = 42.

Table 3. Continued

Abbreviations: amikacin: A, amoxicillin/clavulanic acid: Ax/C, ampicillin: Ap, cefoxitin: C, ceftiofur: Ct, ceftriaxone: Cx, chloramphenicol: Ch, ciprofloxacin: Cp, gentamicin: G, kanamycin: K, nalidixic acid: N, streptomycin: St, sulfisoxazole: Sx, tetracycline: T, and trimethoprim/sulfamethoxazole: Tr/S.

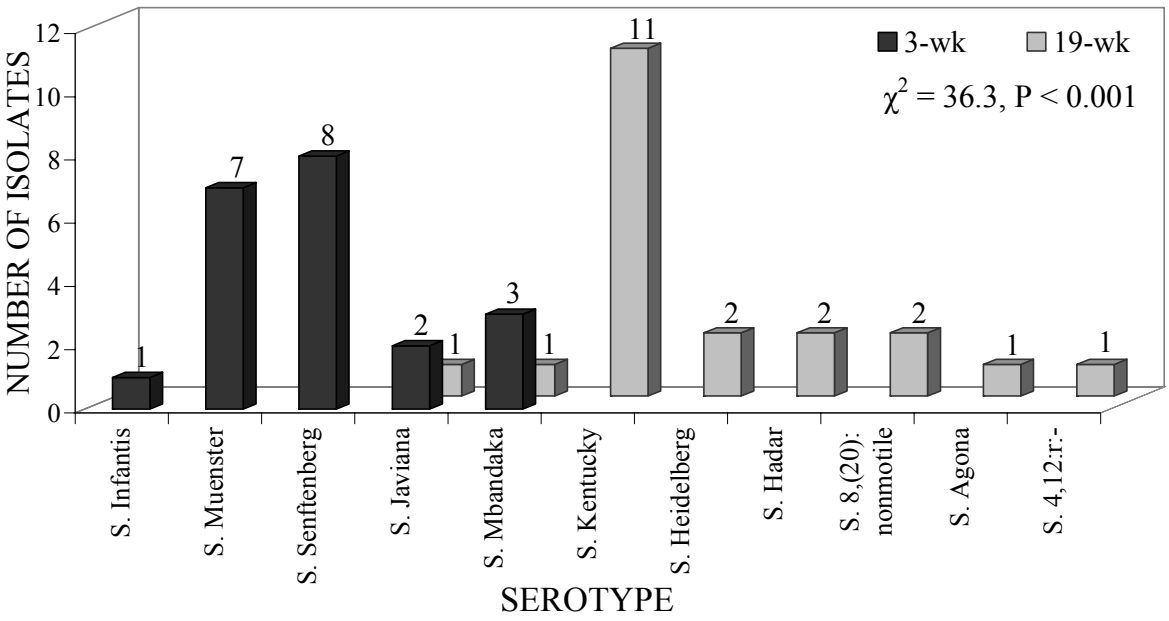

Figure 1. Distribution of *Salmonella* serotypes according to turkey age

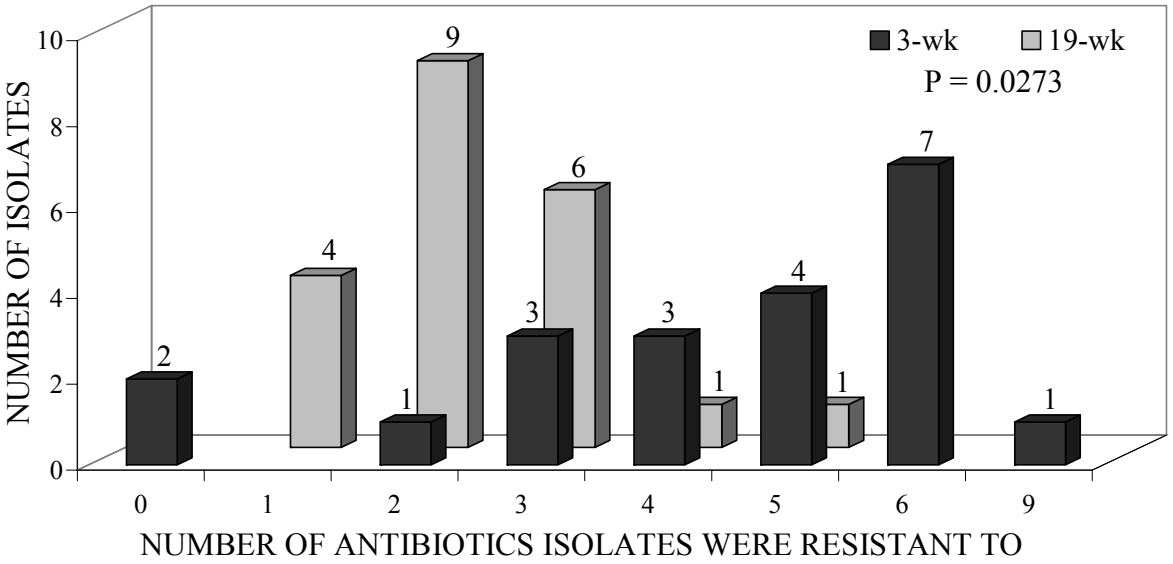

Figure 2. Influence of turkey age on multiple-antibiotic resistance patterns of *Salmonella* serotypes

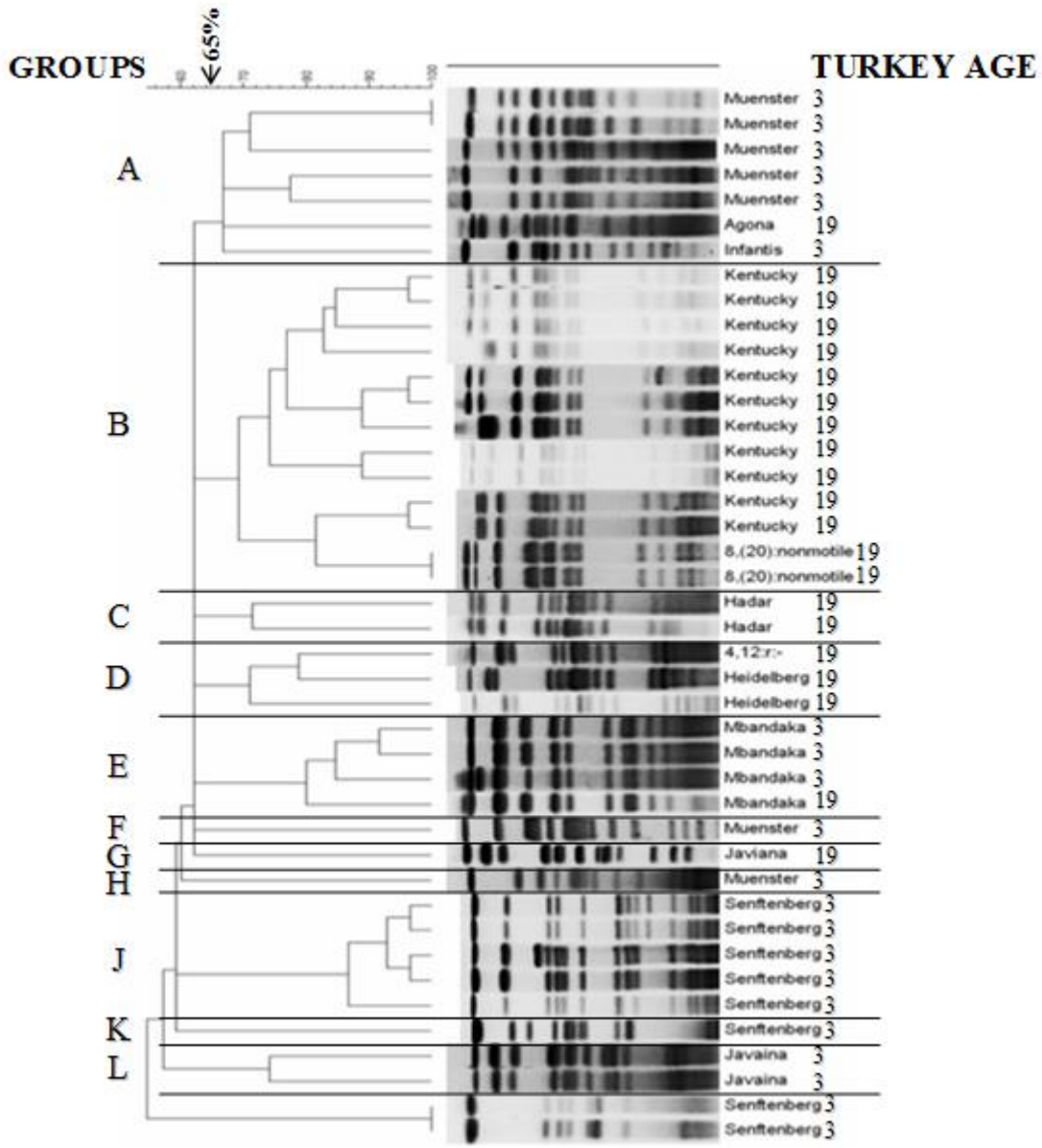

Figure 3. Cluster analysis of the PFGE profiles of *Salmonella* serotypes isolated from turkey fecal or litter samples (the dendogram analysis was band-based and created using the single linkage method. The 12 PFGE groups (A – L) were created based on 65% or greater relatedness between isolates)

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CHAPTER 4

INFLUENCE OF GRAIN PARTICLE SIZE AND INSOLUBLE FIBER CONTENT ON *SALMONELLA* COLONIZATION AND SHEDDING OF TURKEYS FED CORN-SOYBEAN MEAL DIETS[*]

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International Journal of Poultry Science

[*] Use of trade names in this publication does not imply endorsement by the North Carolina Agriculture Research Service or the North Carolina Cooperative Extension Service of the products mentioned, nor criticism of similar products not mentioned.

4.1 ABSTRACT

Previous studies have shown that changes in feed formulation practices can impact the anatomy and physiology of the intestinal tract of poultry. Besides physical changes in the diet, variations in its composition can also alter the avian intestinal microflora. This study was conducted to determine the impact of feeding partially ground corn or insoluble fiber to 0-28 day old turkeys on intestinal development, and its ability to modulate *Salmonella* cecal colonization and fecal shedding. Nicholas toms reared in cage batteries were assigned to 1 of 3 treatment diets: ground corn-SBM (GC, TRT 1), coarse ground corn-SBM (CC, TRT 2), and 4% wood shavings + ground corn-SBM (SC, TRT 3). A 3-strain cocktail of nalidixic acid-resistant *Salmonella enterica* serotypes Hadar, Javaina, and Typhimurium was orally-gavaged into each poult at placement. Cecal and fecal *Salmonella* populations were estimated on days 7, 14, 21, 28, and 1, 14, 28, respectively. Growth performance and intestinal weights and lengths were also measured. The diets had no impact on *Salmonella* cecal or fecal populations. Cecal populations at 7d averaged 5.9, 6.3 and 6.5 log cfu/g for TRT 1, 2 and 3, respectively. At 28d, *Salmonella* cecal populations decreased approximately 3-logs (range: 2.4-3.3 log reduction) across all treatments in comparison to 7d ($P < 0.0001$). At the end of the study body weight, as well as body gain and feed conversion ratio were not impacted by the dietary treatments. However, at 14 days poults consuming the SC diet had lower feed consumption (FC) than those fed the GC and CC diets (231 vs. 243 and 252 g, $P = 0.001$, respectively). The CC and SC diets also produced heavier gizzards than did the GC diets throughout the study. The CC diet resulted in heavier relative gizzard weights at 28d in comparison to the GC and SC diets (30 vs. 28 and 22 g/kg, respectively, $P < 0.0001$). In contrast, the SC treatment reduced the mass of the small intestine relative to body weight,

especially the jejunum. Although dietary inclusion of coarsely ground corn and wood shavings had no detectable influence on *Salmonella* colonization and fecal shedding, it had no adverse effect on growth performance yet improved gizzard and intestinal development, which could have positive effects on gut health.

Keywords: *Salmonella*, turkey, particle size, wood shavings, intestinal development

4.2 INTRODUCTION

Achieving the genetic potential for growth, maintaining health, and food safety at the lowest input costs are the goals of commercial poultry producers throughout the world. Whole grain feeding, dietary enzyme supplementation and pelleting are among the feed manufacturing practices used to achieve these goals. In many countries, feeding whole grains to poultry flocks has become a routine practice to reduce feed manufacturing and handling cost (Svihus et al., 2004), but it may also improve the feed conversion ratio (Plavnik et al., 2002) and nutrient digestibility (Svihus et al., 2004) without adversely affecting weight gain (Bennett et al., 2002; Svihus et al., 2004). Improved resistance to enteric pathogens and reduced carcass contamination due to gut breakage during evisceration may be possible by dietary inclusion of course feed particles that are retained for a longer time period in the gizzard than are finer feed particles (Ferket et al., 2005). Whole grain feeding also increases gizzard weight due to mechanical stimulation by the feed (Engberg et al., 2002; Plavnik et al., 2002; Svihus et al., 2004), which increases reflux motility of the intestine (Duke, 1992). Some studies have also shown that whole or coarsely ground grains will be retained in the gizzard until the particles are reduced to a remarkably small and relatively homogenous size

(Engberg et al., 2002; Svihus et al., 2002). Hetland and coworkers (2002) have shown that 40-70% of the feed particles entering the duodenum are smaller than 100 μm, independently of whether ground or whole cereals have been fed. Moreover, increased gizzard activity has been associated with increased pancreatic enzyme secretion, improved feed flow regulation (Svihus et al., 2004), and stimulation of gastric function such as secretion of hydrochloric acid (Engberg et al., 2002; Engberg et al., 2004). Furthermore, structural properties of feed, grain particle size, and feed formulation can influence intestinal microflora of poultry (Engberg et al., 2002). The increased retention time in the gizzard and a more acidic gizzard pH may not only kill ingested enteric pathogens, but may also increase the fermentation of symbiotic bacteria in the crop that act as seed stock to colonize the lower digestive tract and competitively exclude pathogens (Engberg et al., 2004).

Foodborne pathogen contamination of poultry and poultry products is an ongoing problem for industry, plus some feeding practices are directly related to pathogen contamination of flocks. For example, an increase of coliform bacteria, indicating potential colonization of enteric pathogens such as *Salmonella*, was observed in the ileum of pellet-fed broiler chickens (Engberg et al., 2002). The same study showed a reduction of lactose-negative enterobacteria and *Clostridium perfringens* when chickens were fed whole wheat. Results from a similar experiment showed that whole wheat feeding reduced *Salmonella* in gizzard and ileal contents of broilers (Bjerrum et al., 2005).

The structure of the feed itself can also influence the microflora of the intestinal tract of broiler chickens. Bjerrum and coworkers (2005) demonstrated that the prevalence of *Salmonella* positive gizzards decreased after whole wheat supplementation to a broiler diet. Similar results were reported by Engberg et al. (2002) who showed that the populations of

lactose-negative bacteria decreased after whole wheat feeding. Additionally, Bennett and coworkers (2002b) evaluated the effect of feeding whole barley on turkey performance and concluded that feeding up to 20% whole barley had little or no effect on weight gain and feed efficiency. The authors also observed an improvement in skeletal health and livability; however, characterization of the intestinal microflora was not done. Although the duration of production is considerably longer for turkeys than broilers and their nutrition and management requirements are different, we hypothesize that particle size and structure of feed components could also influence *Salmonella* colonization in turkeys. Therefore, the main objective of this study was to explore the effect of corn particle size (coarse vs. fine) and insoluble fiber content of the diet (addition of wood shavings to a finely ground corn diet) on *Salmonella enterica* colonization, gastrointestinal tract development, and growth performance of turkey toms from 1 to 28 d of age.

4.3 MATERIALS AND METHODS

4.3.1 Bird Husbandry

Six-hundred and twenty-four 1 day-old commercial Nicholas (Aviagen, Huntsville, AL) male turkeys obtained from a commercial hatchery (Goldsboro Milling Co., Goldsboro, NC) were weighed, neck-tagged and orally gavaged with 1 ml (6×10^9 colony forming units – cfu) of a cocktail of *Salmonella enterica*, as described below, before being randomly assigned to 24 experimental cages of two Alternative Design Batteries (Wilveco, Billerica, MA) with 26 birds in each cage. Each cage was 55 cm wide, 66 cm long and 45 cm high. During the experiment the room temperature was regulated continuously, with a starting

temperature of 37°C and a final temperature of 24°C at 28 days. Feed and water were given *ad libitum*.

4.3.2 Experimental Design and Diets

The experimental design consisted of three dietary treatments, each with eight replicate pens of 26 turkey poults. Over the entire experimental period (1-28 d), all turkeys were fed a corn-based diet (Table 1) in mash form. The experimental diets were formulated using least-cost linear programming to meet or exceed NRC (1994) nutrient requirements. The corn included in the feed formulas was either ground fine for experimental treatments GC (ground corn) and SC (wood shavings, ground corn), respectively, or ground coarse for experimental treatment CC. The SC treatment also included 4% (w/w) particles of soft pine shavings. The corn used to prepare the fine mash diet was ground in a hammermill (Bliss Industries Inc., Ponca City, OK) equipped with a 3-mm screen and had a final average particle size of 560 μ. The corn used in the coarse mash diet was prepared by grinding through a 4-mm screen with subsequent sieving in a 60 in. separator (Southern Wire Cloth, Vibrecon Division, Tulsa, OK) using a 16-mm sieve which resulted in a final average particle size greater than 3000 μ. All other ingredients, including the wood shavings, were ground using a 3-mm screen and had a final average particle size of 600 μ. The feed did not contain antimicrobials or coccidiostats.

4.3.3 Bacterial Strains and Inoculum

A cocktail of *Salmonella enterica* subspecies *enterica* serovar Typhimurium, Hadar and Javaina was used as the inoculum. For the purpose of this document, serovars of

Salmonella enterica subsp. *enterica* will be referred to as *Salmonella* accompanied by the serovar name (i.e. *Salmonella* Typhimurium or *S.* Typhimurium). The strains had been previously isolated from turkey feces (Hadar and Javaina) and chicken cecal content (Typhimurium) and presented a natural resistance to nalidixic acid (NAR). These *Salmonella* isolates were serotyped by the NVSL (National Veterinary Service Laboratories, Animal and Plant Health Inspection Services, United States Department of Agriculture, Ames, IA). To prepare the inoculum, the 3 strains were grown separately overnight at 37°C in brain-heart infusion (BHI) broth (Oxoid, Ogdensburg, NY) supplemented with nalidixic acid (NA, Sigma, St. Louis, MO) at a final concentration of 1000 ppm. The cultures were then mixed together and serially diluted in buffered peptone water (BPW, Oxoid, Ogdensburg, NY) to a final concentration of 6×10^9 cfu/ml. The cell count was determined by direct plating on BHI agar plates supplemented with 1000 ppm NA, after overnight incubation at 37°C. Negative controls were used for all plating procedures to ensure that the media had been properly sterilized.

4.3.4 Data Collection

At each sampling time, 2 poults were randomly chosen from each cage, weighed and euthanized by cervical dislocation. The abdomen was opened and the proventriculus, gizzard, small intestine (duodenum, jejunum and ileum) and ceca were collected. The contents of the gizzards and proventriculus were flushed with sterile deionized (DI) water for pH determination and subsequently the weight of both organs was recorded separately. After gizzard weights were recorded, gizzards were stored in icy water for 24 hours and then the glycocalyx lining was peeled from each gizzard and weighed. Ceca were aseptically removed

immediately after the poults were euthanized, weighed, placed in sterile filtered stomacher bags (Spiral Biotech Inc., Norwood, MA) and stored on ice for ca. 30 minutes before being cultivated for *Salmonella* isolation and enumeration. Cultivation for *Salmonella* was conducted immediately after sampling. The small intestine segments (duodenum, jejunum and ileum) and ceca from a third bird were collected and weights and lengths measured. Samples were taken at day 7, 14, 21, and 28. Gizzard lining weight was not determined at day 7. Fecal samples were collected at day 1, 14 and 28 and cultivated for *Salmonella* to assure that turkeys were not only colonized by *Salmonella* but were also shedding the bacterium. Ten grams of fresh fecal samples were collected beneath each cage. Twelve hours before fecal collection the pens were cleaned and all old fecal material removed. Turkeys were inspected daily and birds with visual health problems or poor body condition were removed, weighed and euthanized by cervical dislocation. All mortality was weighed so that an appropriated adjustment of feed conversion ratio (FCR) could be made. Feed consumption by cage and individual bird body weight (BW) were recorded at 1, 7, 14, 21, and 28 days of age.

4.3.5 Analytical Methods

At day of placement, meconium samples from all poults were collected and examined for salmonellae using the most probable number procedure described by Santos et al. (2005). Meconium samples were also processed using the direct plating method to isolate any nalidixic acid resistant (NAR) bacteria. The samples were placed in separate 7 x 12 inch (17.78 x 30.48 cm) sterile filtered stomacher bags (Spiral Biotech Inc., Norwood, MA) followed by the addition of BPW to each bag at a 1:10 dilution. The bags were then

homogenized for one minute using a stomacher (IUL Instruments, S.A., Barcelona, Spain). After homogenization, samples were serially diluted in BPW and direct plated onto BHI+NA plates to assure that poults were not contaminated with NAR bacteria. Plates were incubated for 24 hours at 37°C. Fecal samples collected thereafter and pooled cecal samples were processed for NAR-*Salmonella* isolation using the direct plating method as described above.

Gizzard and proventriculus digesta pH was measured separately immediately following sampling. After being flushed with DI water (ca. 1:10 dilution, content:water ratio) into sterile Whirl-Pak bags (Fisher Scientific International, Bohemia, NY), suspensions of proventriculus and gizzard contents were manually mixed by shaking for 1 minute. The pH probe (Fisher Scientific International, Bohemia, NY) was then inserted direct into each bag and the pH recorded (Fisher Scientific International, Bohemia, NY). The probe was washed using sterile DI water between readings.

The length of the small intestine was measured for each segment as defined by duodenum (from the gizzard to pancreatic and bile duct), jejunum (from the bile duct to Meckel's diverticulum), ileum (from the Meckel's diverticulum to ileo-cecal-colonic junction), and ceca (Samanya and Yamauchi, 2002). Each segment was placed on a ruler and the length (centimeters) recorded. Intestinal segment weights (grams) were recorded after its digesta content had been manually removed. Intestinal weights and lengths were calculated relative to live bird body weight (kilograms) (Bjerrum et al., 2005).

4.3.6 Statistical Analysis

Statistical analysis of results was performed using the general linear model procedure (Proc GLM) of SAS (SAS Institute, 1996) according to the following general model: $Y_{ijk} = \mu$

$+ \alpha_i + \beta_j + (\alpha\beta)_{ij} + E_{ijk}$, where Y_{ijk} was the observed dependent variable; μ the overall mean; α_i the dietary treatment effect; β_j the block effect; $(\alpha\beta)_{ij}$ the interaction between dietary treatment and block; and E_{ijk} the random error. Replicate pens of 26 birds served as experimental units. When treatment effects were identified to be significant by the F-test ($P < 0.05$), the treatment means were separated by the least-square-means (lsmeans) function of SAS with a confidence of $P < 0.05$. Before statistical analysis, all cell count (cfu) data were transformed to the base-10 logarithm and the intestinal measurement data were transformed relative to body weights. Finally, correlation between *Salmonella* cecal population, relative ceca weight, and gizzard and proventriculus pH was accomplished using the correlation procedure of SAS (SAS Institute, 1996) and the results were expressed as r values.

4.3.7 Animal Ethics

The experiments reported herein were conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry and euthanasia practices were performed with full consideration of animal welfare.

4.4 RESULTS

Body weights, which ranged from 794 g (CC) to 829 g (GC) at 28 d, were not affected by the dietary treatments throughout the trial (Table 2). From 7 to 14 d, supplementation of insoluble fiber to the diet (SC) resulted in the lowest feed intake and the CC diet had the highest (169.3 and 191.7 g, $P < 0.01$, Table 3). Although there was no significant treatment effect observed for feed consumption between 1 and 7 d, dietary

inclusion of either 160 g of insoluble fiber as wood shaving per kg of corn-based diet (SC) or coarsely ground corn (CC) resulted in significantly better feed conversion than finely ground corn (GC) (1.05, 1.08 vs. 1.16 g/g, $P < 0.001$, respectively) during the first 7 days post-hatch (Table 4). Furthermore, contrast analysis revealed that turkeys fed the diet supplemented with wood fiber had a significantly better 1 to 28 d FCR than those non-supplemented (1.34 vs. 1.45 g/g, $P = 0.0443$).

Particle size of the corn and inclusion of shavings into the diet had a highly significant impact on relative gizzard weights, but not on gizzard digesta pH which averaged 3.3 across treatments. At day 7, the birds fed diets containing coarse ground corn (CC) and wood shavings (SC) had about 18.1% greater gizzard mass relative to body weight than those fed the diet containing the finely ground corn (GC). Moreover, this difference in relative gizzard weight between GC and CC or SC increased to 26.2% and 24.7% by 14 d and 21 d, respectively. However, by day 28 the relative gizzard weights of birds fed CC and SC diets were 36% and 20.5% greater than those fed the GC diet, respectively. As observed with relative gizzard size, poults fed the CC diet had significantly larger gizzard lining weights relative to body weight at day 28 as compared to those fed the SC and GC diets (4.4 vs. 3.9 and 3.8 g/kg, respectively; $P < 0.05$). Interestingly, when gizzard lining weights were expressed relative to the total gizzard weight (Table 6), lining weights were not significantly different between the three dietary treatments ($P > 0.05$). This finding indicates that both lining and muscle developed at a similar rate. Relative proventriculus weights and pH averaged 6.56 g/kg and 3.4, respectively, regardless of dietary treatment (data not shown).

The dietary treatments had no effect on relative intestinal lengths throughout the experiment. At day 28, the mean lengths were 26.5, 56.0, 61.7, 19.6 cm/kg of body weight

for duodenum, jejunum, ileum and ceca, respectively (Table 7). In contrast, jejunum weight was affected by the dietary treatments (Table 8). At day 14, birds fed the CC diet had significantly greater jejunum weights than those fed GC or SC diets (P = 0.01). By day 28, however, relative jejunum weight of birds fed the SC diet were significantly (P = 0.01) lower than those fed either GC or CC. Similar treatment effects were observed with respect to the relative size of the whole small intestine (sum of the relative weights of duodenum, jejunum and ileum) (Table 9). Poults fed the diet containing wood shavings (SC) had lower relative small intestinal weights than those fed the GC or CC diets (7.6 to 9.0 g/kg lighter, respectively, P = 0.01).

The results of the *Salmonella* population estimates in the cecal and fecal samples are summarized in Table 10. Pooled meconium samples collected from 3 individual cages (one cage per treatment) were positive for *Salmonella* at day of placement. These *Salmonella* isolates were further tested and found to be sensitive to nalidixic acid. Moreover, there was no significant difference in the population of NAR-*Salmonella* organisms detected between the three treatment groups. Although not influenced by dietary treatment, average cecal *Salmonella enterica* populations in the poults decreased 3-logs from day 7 to 28 (6.29 vs. 3.48 log/g, P < 0.0001). Fecal *Salmonella* populations were not significantly affected by the dietary treatments and averaged 4.7 log from day 7 to 28.

Relative ceca weight was inversely correlated to cecal *Salmonella* populations (Table 11). Additionally, although not very strong, the positive relationship between cecal populations and proventriculus pH (r = 0.34, P = 0.001) suggest that acid exposure in the proventriculus may be an important inhibitor of *Salmonella* colonization in the ceca. Although there was no significant correlation observed between cecal *Salmonella* population

and gizzard pH ($P > 0.05$), a positive correlation between the digesta pH in the proventriculus and the digesta pH in the gizzard was statistically significant ($r = 0.44$, $P < 0.0001$).

4.5 DISCUSSION

Feeding coarsely ground corn or increasing the insoluble fiber content of the diet by 4% had a greater effect on growth performance of turkeys during the starting phase of the trial (1-14 d) than during the last two weeks of the trial (14-28 d). Feeding coarse ground corn or wood shavings improved the FCR during the critical 7 d post-hatch period (Table 4, $P = 0.001$). The positive effect of the SC diet continued through day 14 as the SC poults consumed less feed than birds on the other two treatments, without adversely affecting body weight (Table 2, $P = 0.001$) and by the end of the experiment (28 d) the SC fed birds had about 8% lower cumulative FCR than the birds fed non-supplemented diets. These findings agree with the work reported by Yasar (2003) who showed that broiler chickens fed whole or coarsely ground wheat from 1 to 28 days of age had better growth performance when compared to birds fed finely ground wheat. Development of the intestinal tract is probably one of the main reasons for the better performance of the birds fed diets with large particle size and insoluble fiber content in comparison to the ground corn/SBM treatment group (Jones and Taylor, 2001).

Feeding coarsely ground corn and wood shavings also increased the relative gizzard weights by day 28 by 35% and 25%, respectively. Similar results have been observed for turkey toms fed whole barley (Bennett et al., 2002a) and broiler chickens (Bennett et al., 2002b) fed whole wheat, where gizzard weights increased 34% and 37%, respectively. Other

studies also reported increased gizzard weights when broilers were fed whole wheat (Svihus and Hetland, 2001; Svihus et al., 2002; Plavnik et al., 2002).

The gizzard is an important organ in the digestion process and greatly influences the normal intestinal motility (Duke, 1992). It influences digestion and absorption of nutrients by increasing digesta retention via periodic reverse peristalsis (Moran, 1982). Furthermore, the rate of the cloaca-ceca reflux, a low amplitude colonic anti-peristalsis that conveys the urethral secretion along the epithelial surface of the rectum into the ceca where uric acid and nitrogen is converted into microbial biomass, is dependent upon the activity of the upper-intestine motility, particularly of the gizzard (Duke, 1992). Therefore, an improvement in gizzard activity would have a beneficial effect in general bird digestion and absorption of nutrients.

The use of whole grains also contribute to the optimization of enteric health and digestive capacity in broilers by improving the development and function of the foregut organs such as the crop, proventriculus and gizzard (Taylor and Jones, 2004). For example, whole grain feeding not only increases the size of the gizzard musculature because of greater grinding activity, but it also increases the volume and retention time of ingesta in the organ. Coarse particles, such as whole wheat or whole barley, increase gizzard motility (Bennett et al., 2002a; Bennett et al., 2002b; Plavnik et al., 2002; Svihus et al., 2002). Retention time of feed in the gizzard is also increased after feeding coarser rations (Svihus et al., 2002) because feed particles need to be reduced to a small and homogenous size, averaging 100 μm, before they leave the gizzard (Hetland et al., 2002). Increasing retention time in the gizzard exposes the contents to greater peptic digestion, which is particularly important for enhancing protein digestion efficiency in the small intestine. Bjerrum et al. (2005) reported that feeding whole

grains to broiler chickens caused a significant reduction in the gizzard content pH, probably due to mechanical stimulation of the proventriculus which leads to increased hydrochloric acid production. Each time the gizzard contracts, some of its contents are refluxed back into the proventriculus for further exposure to peptic secretions of pepsin and hydrochloric acid. Pepsin is an endopeptidase that cleaves proteins into multiple peptide fragments. These peptide fragments are then further digested by pancreatic exopeptidases such as trypsin and chymotrypsin. Whole grain feeding also increases pancreas and liver secretions (Svihus et al., 2004). Therefore, the benefit of enhanced gizzard function is better foregut digestion of protein and fat resulting in less available nutrients to be used as substrates in the hindgut by pathogenic organisms such as salmonellae and clostridia.

In addition to improving the efficiency and capacity of digestion, dietary inclusion of coarse material that promotes gizzard activity may influence the composition and populations of intestinal microflora by suppressing the colonization of enteric pathogens which compete for the same nutrients as the host and promoting the colonization of symbiotic organisms. For instance, increased gizzard retention discourages the colonization of ingested pathogens sensitive to acidic conditions. Conversely, increased gizzard retention may promote the growth of fermentative microorganisms in the crop (e.g. lactobacilli) which are more resistant to the lower pH in the gizzard environment, thereby seeding the intestine with symbiotic bacteria. However, Svihus et al. (2002) did not detect any changes in the passage rate of feed through the gizzard after feeding whole grains. Thus, the inverse relationship of dietary particle size and intestinal colonization of microbial pathogens is less likely to be due to prolonged exposure to hydrochloric acid in the gizzard and more likely due to improved protein and fat digestibility in the foregut.

In the present study, cecal colonization and fecal shedding of NAR-*Salmonella* were not affected by the dietary treatments. Other recently completed studies documented that feed particle size did not influence cecal *Salmonella* colonization of broilers fed whole wheat (Bjerrum et al., 2005) or coarsely ground corn (Huang et al., 2006). Mikkelsen et al. (2004) also observed no significant effects of feed particle size and feed form on *Salmonella* death rate in the ceca of pigs. Several factors may have influenced the outcome of the present study, including the high inoculation dose used (6×10^9 cfu/bird) and the age at which the poults were challenged. The poults received a very high dose of *Salmonella* at a very young age before introduction and adaptation to the dietary treatments. In addition, the duration of the experiment may have been insufficient to detect a positive response. Although there was no significant dietary effect on gizzard and proventriculus digesta pH, cecal *Salmonella* populations were significantly correlated to the proventriculus pH ($r = 0.34$, $P = 0.001$), showing that acid exposure in the proventriculus may interfere with the colonization of the ceca by *Salmonella*. Santos et al. (2005) reported a significant linear correlation between *Salmonella* population and pH in litter collected from commercial turkey houses. Similarly, Bjerrum et al. (2005) reported a reduction of *Salmonella* populations in the gizzard contents when the pH was lowered by feeding whole wheat to broilers. Even though the correlation between *Salmonella* cecal population and gizzard pH was not significant, a positive relationship between the pH of the gizzard and proventriculus digesta was detected ($r = 0.44$, $P < 0.0001$).

A significant inverse correlation between cecal *Salmonella* population and relative ceca weight was observed ($r = -0.34$, $P = 0.001$). Similar results were reported by Santos (2006) who found that turkeys fed diets high in non-starch polysaccharide (NSP) had lower

cecal *Salmonella* populations and increased relative ceca weight. The author suggested that high dietary NSP (fiber) content may have increased commensal microbial fermentation in the ceca thus increasing short-chain volatile fatty acid (SCFA) production which suppressed *Salmonella* colonization. SCFA decreases cecal pH and creates an inadequate environment for the growth of some pathogenic microorganisms like *Salmonella*. Furthermore, SCFA produced by increased microbial fermentation in the lower intestinal tract have important metabolic functions that control microbial populations in the gastrointestinal tract. SCFA stimulate intraepithelial lymphocytes and natural killer cells (Ishizuka et al., 2004) which enhances the immunocompetence of the host animal (Lan, 2004) and suppresses the colonization of pathogens (Bertschinger et al., 1978; Lowry et al., 2005). SCFA-producing commensal bacteria also compete with pathogens for available nutrients and attachment sites in the intestinal tract (Simon et al., 2004), therefore competitively excluding *Salmonella*.

Another explanation for the inverse association between cecal *Salmonella* colonization and relative ceca weight would be that the natural microflora of the birds with higher cecal *Salmonella* colonization are suppressed, thereby compromising the normal function of the ceca and causing hypotrophy of the organ. This is consistent with the findings of Tannock and Savage (1976) who showed that the ceca of germ-free mice challenged with *Salmonella* Typhimurium were significantly smaller (% of body weight) than the ceca of unchallenged germ-free mice. The authors also showed that once mice were either vaccinated with heat-killed *Salmonella* or were exposed to indigenous bacteria prior to challenge, the cecum was of normal size. The authors suggested that there may be a synergistic mechanism between the influence of the normal microflora and the host's immune response.

Intestinal lengths were not affected by the treatment diets; however, jejunum and total small intestine weights were reduced for turkeys fed diets supplemented with 4% wood shavings. At 28 days of age, the jejunum of turkeys fed the diet supplemented with wood shavings (SC) weighed 17% less on a relative basis than the other two dietary treatments (GC and CC) ($P = 0.01$). The metabolic cost involved in maintaining the gastrointestinal tract accounts for about 30% of the total body metabolic rate (Aiello, 1997). Thus, larger digestive organs may compromise lean tissue growth because more protein synthesis and energy could be directed toward organ growth (Lan, 2004). In agreement, the performance data in this study showed that the SC diet reduced the FCR which is likely associated with a reduction in the relative mass of the small intestine.

4.6 CONCLUSIONS

1. Dietary inclusion of coarse ground corn or wood shavings improved FCR during the critical 7 d post-hatch period. Additionally, the positive effect of dietary supplementation of wood shavings continued through day 14 as the SC-fed poults consumed less feed than did the poults fed the other two treatments without adverse effects on body weight.
2. Dietary inclusion of wood shavings improved by 8% the 1 to 28 d FCR of turkey poults.
3. Supplementation of wood shavings to the diet decreased the relative small intestine and jejunum weight, which likely is associated with the reduction in feed conversion ratio in the SC-treatment group, as less energy is required to maintain the gastrointestinal tract.
4. Diets formulated with coarse ground corn and wood shavings increased the relative gizzard weight and possibly improved digestion and absorption of nutrients, as the

gizzard is an important organ in the digestion process and greatly influences the normal intestinal motility.

5. Cecal *Salmonella* populations were positively correlated to proventriculus pH, indicating that lower proventriculus pH can contribute to reduced cecal *Salmonella* colonization. Additionally, there was a positive relationship between the pH of the proventriculus and the pH of the gizzard digesta.

ACKNOWLEDGMENT

This study was supported by the USDA Initiative for Future Agriculture and Food Systems (IFAFS) grant. The authors wish to thank Annette Israel, Jamie Warner, Jean de Oliveira, Ondulla Foye, Renee Plunske, Mike Mann, Robert Neely and Gene Alborne for their technical assistance during this study. Appreciation is also extended to Carl Whisenant for helping with the preparation of the coarse ground corn and wood shavings used in this experiment, and to Paul Otto for helping with the feed mixing..

4.7 TABLES

Table 1. Nutrient composition of turkey diets containing different particle sizes of corn and supplemented with wood shavings as a source of insoluble fiber

INGREDIENTS	GC (TRT 1)[1]	CC (TRT 2)[2]	SC (TRT3)[3]
		(%)	
Corn, Grain	52.72	52.72	46.48
SBM – 48%	30.00	30.00	30.00
Gluten Meal	5.00	5.00	5.00
Soybean Oil	0.00	0.00	2.18
Poultry Meal	8.00	8.00	8.00
Wood Shavings	0.00	0.00	4.00
DL-Methionine	0.08	0.08	0.09
L-Lysine HCl	0.42	0.42	0.44
Limestone	1.26	1.26	1.24
Vit/Min Premix	2.53	2.53	2.57
TOTAL	100	100	100
		Calculated Analysis	
Kcal ME/g	2.92	2.92	2.90
Crude Protein (%)	27.53	27.53	27.01
Fat (%)	3.47	3.47	5.42
Crude Fiber (%)	2.55	2.55	6.34
Calcium (%)	1.20	1.20	1.20
Available P (%)	0.60	0.60	0.60
Sodium (%)	0.17	0.17	0.17
Lysine (%)	1.60	1.60	1.60
Methionine (%)	0.55	0.55	0.55
Cysteine (%)	0.42	0.42	0.41
		Chemical Analysis[4]	
Dry Matter (%)	92.55	92.55	92.83
Crude Protein (%)	27.51	27.51	28.37
Gross Energy (kcal/kg)	4271.23	4271.23	4326.51
Fat (%)	2.65	2.65	4.57
Ash (%)	7.35	7.35	7.72
Fiber Total (%)	25.02	25.02	27.23
Insoluble (%)	9.02	9.02	15.95
Soluble (%)	16.00	16.00	11.28

[1] Finely ground corn.
[2] Coarsely ground corn.
[3] Wood shavings + finely ground corn.

Table 1. Continued

[4] Chemical analysis (*dry matter basis*): (1) Crude protein determined using Kjeldahl automatic analyzer (Kjeltec Auto 1030 Analyser, Tecator, Sweden), (2) Gross energy determined using bomb calorimetry (IKA Calorimeter System C5000 control, IKA® Werke Labortechnik, Staufen, Germany), (3) Fat determined by ether extraction (Labconco Corporation, Kansas City, MO) method, (4) Ash determined by muffle oven (Thermolyne, Sybron Corporation, Dubuque, IA) method, and (5) Dietary fiber analysis was conducted by Intertek Agri Services Food Agricultural Testing Laboratory (St. Rose, Louisiana) using the AOAC method for soluble, insoluble and total dietary fiber analysis.

Table 2. Effect of grain particle size and level of insoluble dietary fiber on body weight of turkeys fed corn/SBM-based diets

TREATMENT	7 d	14 d	21 d	28 d
		(g)		
GC[1]	119.2	264.8	501.0	829.3
CC[2]	114.5	269.1	501.3	794.0
SC[3]	117.8	259.3	498.3	823.6
P-VALUE	0.2132	0.5245	0.9415	0.5831
SEM(12)[4]	1.801	5.940	6.795	25.224

[1] GC: finely ground corn.
[2] CC: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(12): standard error of the mean with 12 degrees of freedom.

Table 3. Effect of grain particle size and level of insoluble dietary fiber on periodic feed consumption of turkeys fed corn/SBM-based diets

TREATMENT	1 to 7 d	7 to 14 d	14 to 21 d	21 to 28 d	1 to 28 d
			(g)		
GC[1]	69.5	173.7[b]	304.4	530.5	1078.1
CC[2]	59.9	191.7[a]	321.4	510.4	1083.4
SC[3]	61.6	169.3[c]	296.7	488.0	1015.5
P-VALUE	0.1170	0.0023	0.3802	0.2431	0.1739
SEM(12)[4]	2.674	3.668	12.327	16.840	26.514

[1] GC: finely ground corn.
[2] CC: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(12): standard error of the mean with 12 degrees of freedom.
[a,b, c] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 4. Effect of grain particle size and level of insoluble dietary fiber on periodic feed conversion ratio of turkeys fed corn/SBM-based diets

TREATMENT	1 to 7 d	7 to 14 d	14 to 21 d	21 to 28 d	1 to 28 d
			(g/g)		
GC[1]	1.16[a]	1.20	1.29	1.68	1.41
CC[2]	1.08[b]	1.25	1.38	1.76	1.48
SC[3]	1.05[b]	1.16	1.20	1.53	1.34
P-VALUE	0.0008	0.7511	0.1880	0.3181	0.0695
SEM(12)[4]	0.015	0.052	0.049	0.105	0.039

[1] GC: finely ground corn.
[2] CC: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(12): standard error of the mean with 12 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 5. Effect of grain particle size and level of insoluble dietary fiber on relative gizzard and gizzard lining weight of turkeys fed corn/SBM-based diet

TREATMENT	7 d	14 d		21 d		28 d	
	RGW[5]	RGW	RGLW[6]	RGW	RGLW	RGW	RGLW
	(g/kg)						
GC[1]	45.16[b]	28.73[b]	4.55[b]	27.26[b]	4.93	22.03[c]	3.77[b]
CC[2]	54.57[a]	36.48[a]	5.55[a]	34.11[a]	4.93	29.93[a]	4.43[a]
SC[3]	52.15[a]	36.04[a]	5.40[a]	33.88[a]	5.22	27.57[b]	3.93[b]
P-value	0.0135	<0.0001	0.0066	<0.0001	0.9651	<0.0001	0.0161
SEM (36)[4]	2.2140	0.8586	0.2226	0.7832	0.1896	0.6465	0.1223

[1] GC: finely ground corn.
[2] CC: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(36): Standard error of the mean with 36 degrees of freedom.
[5] RGW: relative gizzard weight (grams of tissue per kilogram of live body weight).
[6] RGLW: relative gizzard lining weight (grams of tissue per kilogram of body weight).
[a,b, c] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 6. Effect of grain particle size and level of insoluble dietary fiber on gizzard lining weight relative to total gizzard weight[1] of turkeys fed corn/SBM-based diets

TREATMENT	14 d	21 d	28 d
		(g/g)	
GC[2]	0.14	0.16	0.15
CC[3]	0.15	0.16	0.15
SC[4]	0.17	0.16	0.16
P-VALUE	0.3684	0.5941	0.4622
SEM(36)[5]	0.0061	0.0059	0.0051

[1] Relative gizzard lining weight: gizzard lining weight/gizzard weight.
[2] GC: finely ground corn.
[3] CC: coarsely ground corn.
[4] SC: wood shavings + finely ground corn.
[5] SEM(36): standard error of the mean with 36 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 7. Mean relative intestinal weight[1] and length[2] as a function of body weights of small intestine segments and ceca of turkeys fed corn/SBM-based diet[3]

AGE	DUODENUM	JEJUNUM	ILEUM	CECA
		(g/kg)		
7	17.54	29.88	25.48	9.03
14	15.74	21.96	22.61	10.81
21	13.19	18.25	17.86	11.76
28	11.96	17.059	17.86	13.06
		(cm/kg)		
7	109.113	251.526	249.881	71.370
14	61.882	128.477	139.750	42.719
21	39.533	83.271	93.686	26.445
28	26.521	56.004	61.738	19.622

[1] Relative intestinal weight: grams of tissue/kg of body weight.
[2] Relative intestinal length: cm of tissue/kg of body weight.
[3] Average means across dietary treatments, since no significant treatment effects were detected ($P > 0.05$).

Table 8. Effect of grain particle size and level of insoluble dietary fiber on relative jejunum weight (g of tissue/kg of body weight) of turkeys fed corn/SBM-based diet

TREATMENT	7 d	14 d	21 d	28 d
		(g/kg)		
GC[1]	29.13	20.81[b]	18.47	18.28[a]
CC[2]	31.67	24.34[a]	19.03	17.72[a]
SC[3]	28.85	20.73[b]	17.24	15.18[b]
P-value	0.4976	0.0112	0.1031	0.0110
SEM(36)[4]	1.2119	0.9127	0.5857	0.7286

[1] GC: finely ground corn.
[2] CC: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(36): Standard error of the mean with 36 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 9. Effect of grain particle size and level of insoluble dietary fiber on relative small intestine weight[1] (g of tissue/kg of body weight) of turkeys fed corn/SBM-based diet

TREATMENT	7 d	14 d	21 d	28 d
		(g/kg)		
GC[2]	71.55	57.90	49.78	50.36[a]
CC[3]	75.47	64.84	51.21	48.98[a]
SC[4]	71.73	58.16	46.87	41.31[b]
P-value	0.3767	0.1873	0.3009	0.0116
SEM(36)[5]	1.9494	1.8914	1.4578	1.4957

[1] Small intestine weight is the sum of the duodenum, jejunum and ileum weights.
[2] GC: finely ground corn.
[3] CC: coarsely ground corn.
[4] SC: wood shavings + finely ground corn.
[5] SEM(36): Standard error of the mean with 36 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 10. Effect of grain particle size and level of insoluble dietary fiber on *Salmonella* cecal colonization and fecal shedding of turkeys fed corn/SBM-based diets

TREATMENTS	1 d	7 d	14 d		21 d	28 d	
	Fecal[5]	Cecal	Cecal	Fecal	Cecal	Cecal	Fecal
	log MPN/g[6]	log cfu/g[7]					
GC[1]	3.46	5.92	4.17	4.80	5.65	3.50	4.00
CC[2]	3.46	6.38	4.69	5.84	5.77	3.69	4.84
SC[3]	2.98	6.57	4.95	5.67	5.48	3.26	5.43
P-value	-	0.8938	0.5551	0.0895	0.4749	0.5952	0.0613
SEM(12)[4]	-	0.4122	0.5816	0.1980	0.3350	0.5456	0.1984

[1] C: finely ground corn.
[2] C: coarsely ground corn.
[3] SC: wood shavings + finely ground corn.
[4] SEM(12): Standard error of the mean with 12 degrees of freedom.
[5] *Salmonella* population results represent only one positive pen at placement day.
[6] log MPN/g: base-10 logarithm of the most probable number of *Salmonella* present per gram of turkey fecal samples (meconium).
[7] log cfu/g: base-10 logarithm of the colony count of *Salmonella* present per gram of turkey cecal samples.

Table 11. Pearson correlation coefficients of *Salmonella* cecal population, relative ceca weight, and pH of proventriculus and gizzard of turkey toms fed corn/SBM-based diets

	LOG C[1] × RCW[2]	**LOG C × PV pH[3]**	**LOG C × G pH[4]**	**PV pH × G pH**
r[5]	-0.34	+0.34	+0.13	+0.44
P-value	0.001	0.001	0.193	<0.0001

[1] LOG C: base-10 logarithm of the cell count (cfu) of *Salmonella* present per gram of turkey cecal samples.
[2] RCW: relative ceca weight (grams of tissue per kilogram of body weight).
[3] PV pH: proventriculus pH.
[4] G pH: gizzard pH.
[5] r-VALUE: Person correlation coefficients, n = 96.

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CHAPTER 5

INFLUENCE OF HOUSING SYSTEM, GRAIN TYPE AND PARTICLE SIZE ON *SALMONELLA* COLONIZATION AND SHEDDING OF BROILERS FED TRITICALE- OR CORN-SOYBEAN MEAL DIETS[*]

[*] Use of trade names in this publication does not imply endorsement by the North Carolina Agriculture Research Service or the North Carolina Cooperative Extension Service of the products mentioned, nor criticism of similar products not mentioned.

5.1 ABSTRACT

Salmonella colonization in poultry may be influenced by dietary grain type and particle size. Broilers reared either in a cage-based house (Broilermatic System) or a conventional house (litter floor) from 0-42 d were assigned to 1 of 4 dietary treatments (TRT): 1) ground corn-SBM (C, 560µ), 2) coarse ground corn-SBM (CC, >1700µ), 3) ground triticale-SBM (T, 560µ), and 4) whole triticale-SBM (WT). A 4-strain cocktail of *Salmonella enterica* was orally-gavaged into each chick at placement. Growth performance, cecal and fecal *Salmonella* population, gizzard and proventriculus pH, intestinal size (weight and length), jejunum histomorphometry and carcass yields were measured. *Salmonella* population was estimated using the most probable number (MPN) method. Intestinal size was calculated relative to BW. Statistical significance was considered at a $P < 0.05$. Broilers responded differently to the dietary treatments according to housing system. At 42d, birds reared on litter and fed ground grain had greater BW than those fed course grain (2.87 vs. 2.71 kg, $P = 0.001$), and the cage-reared broilers were heavier when fed ground triticale than corn (2.75 vs. 2.64 kg, $P = 0.01$). Broilers raised on litter had better 1-42d FCR and those raised in the cages (1.71 vs. 1.81 g/g, $P = 0.02$). Independent of housing system, relative eviscerated carcass weights of birds fed T and C were heavier than CC and WT fed broilers (762 vs. 752 g/kg, $P = 0.01$). Generally, jejunum villus area and mucosal depth were larger, whereas the small intestine was lighter and shorter in broilers raised on litter than in cages. Relative gizzard weight of broilers raised on litter was greater than those raised in cages (22.2 vs. 20.8 g/kg). Broilers fed the coarse diets had heavier gizzards than those fed the fine ground diets (23.42 vs. 19.56 g/kg, $P < 0.0001$). Broilers fed triticale-based diets had heavier proventriculus than corn-fed broilers, regardless of housing design (5.9 vs. 6.3 g/kg, P <

0.001). Feeding whole or coarse ground grains decreased cecal *Salmonella* populations in 42d-broilers (3.8, 3.9, 4.4, 4.4 log MPN/g for TRT CC, WT, C, T, respectively, $P < 0.05$). Additionally, 42d-broilers reared on litter had lower *Salmonella* populations than those in cages (3.8 vs. 4.4 log MPN/g, $P < 0.01$). In conclusion, triticale is a good alternative feed ingredient to corn, resulting in improved body weights and reduced *Salmonella* colonization. Broilers raised on litter may have achieved lower cecal *Salmonella* populations than those raised in cages because access to litter apparently modulated the intestinal microflora by increasing competitive exclusion microorganisms which discouraged *Salmonella* colonization.

Key words: *Salmonella*, housing system, grain particle size, triticale, broiler

5.2 INTRODUCTION

Food-producing animals are the main reservoir for several human bacterial pathogens. *Salmonella* species are among some these pathogens, causing thousands of foodborne infections each year by consuming contaminated meat and other products of animal origin (Oosterom, 1991). Although occasionally associated with exposure to pets, reptiles and contaminated water, it has been estimated that approximately 95% of salmonellosis cases are primarily of foodborne origin (Mead et al., 1999). In 2005, there were 6,471 (14.55 per 100,000 population) laboratory-confirmed human *Salmonella* infections in the 10 FoodNet surveillance states (CDC, 2006). *Salmonella* has been closely associated with the consumption of poultry and poultry products (Hoszowski et al., 1996; Byrd et al., 1997) and several human infections likely originated at the poultry production level. Previous

studies have demonstrated that contamination of poultry can occur at any point in the production continuum (Bains and MacKenzie, 1974). The prevention of poultry-related salmonellosis has been a major effort of governmental agencies, processing plants and producers. An important means of preventing human salmonellosis is by preventing the infection of live poultry at the farm level (Porter, 1998).

Competitive exclusion products, probiotics, prebiotics, organic acids and enzymes are often used to reduce or eliminate *Salmonella* and other pathogens associated with the poultry production. The competitive exclusion method was first proposed by Nurmi and Rantala (1973) and consisted of administration of ingesta from the crop and intestinal tract of adult birds to day-old chicks. This method has shown to have a protective effect on *Salmonella* colonization of broilers (De Oliveira et al., 2000). More recently, the use of defined microbial species to protect poultry from pathogen colonization has been widely accepted and has become known as the general term "probiotics". *Lactobacillus* and *Bifidobacterium* species have been used most extensively as probiotics in humans, whereas species of *Bacillus*, *Enterococcus,* and *Saccharomyces* have been most commonly used in livestock (Salminen et al., 1998). Improvement of productive performance and feed conversion ratio (FCR) along with decreased intestinal *Salmonella* Typhimurium colonization and bird mortality have been reported following the administration of probiotics to broiler chickens (Corrier et al., 1995). Prebiotics are dietary components that are not digested by the host, but they stimulate the growth and/or activity of one or a limited number of beneficial commensal bacteria in the gastrointestinal tract (GIT), predominantly those that produce short-chain fatty acids (SCFA) (Lan, 2004). Since bacterial species differ from each other in relation to their substrate preferences and growth requirements, bacterial community structure is very much dependent

upon the diet as the ultimate source of substrates for its metabolism (Savory, 1992; Wagner and Thomas, 1987). Therefore, both probiotics and prebiotics can encourage the colonization of commensal bacteria at the lower intestinal tract.

Dietary inclusion of cereals has been shown to influence the ecology of the intestinal tract of poultry, including *Salmonella* colonization. Dietary inclusion of whole wheat can decrease intestinal *Salmonella* colonization in broilers (Bjerrum et al., 2005). Similar effects were observed with turkeys fed wheat- or triticale-based diets (Santos, 2006). Altering feed structure by changing grain particle size is another approach to control salmonellae in the poultry industry (Bjerrum et al., 2005; Huang et al., 2006). Improvement in feed efficiency (Plavnik et al., 2002; Lentle et al. 2006) and nutritive value of the feed (Svihus et al., 2004) has been reported when broilers were fed whole wheat-based diets. Similar results were observed in turkeys fed whole wheat (Bennett et al., 2002a), although growth was only minimally affected by the dietary treatments.

Besides affecting growth performance, changing feed structure or form can influence *Salmonella* infection, as observed in some studies with broiler chickens. Bjerrum et al. (2005) reported that broilers fed pelleted feed had a higher *Salmonella* population in the gizzard than those consuming pelleted feed supplemented with whole wheat. Similarly, Huang and coworkers (2006) demonstrated an increased incidence of *Salmonella* Typhimurium in the gizzard and cecal contents of broilers fed finely ground as opposed to coarsely ground mash feed. Engberg et al. (2002) observed that feeding broilers pelleted feed resulted in a greater number of ileal coliform bacteria, an indicator of potential pathogens, than those fed mash feed. Moreover, the physical characteristics of the feed, such as particle size, can influence intestinal pH of the digestive tract of broilers (Svihus et al., 2004; Huang

et al., 2006), which may influence *Salmonella* colonization. In conclusion, feed structure may influence *Salmonella* colonization by changing the gastrointestinal ecology of broilers.

Disposal of poultry litter and preventing the recycling of infectious pathogens is also a great concern for the poultry industry. Poultry litter consists of manure, a bulking agent to absorb moisture (i.e. wood shavings, straw, and peanut hulls), and other components (i.e. feathers and soil) (Kelley et al., 1994). To address the concerns about litter-rearing systems, non-litter systems are being considered for the broiler and turkey industry. The Broilermatic Cage System is a non-litter system that allows broilers to be raised in large cages with plastic-covered nylon floors. These trampoline-like floors are supported every 11 cm with metal bars covered with plastic strips running the length of the cage, and three crossbars running the width of the cage. This design provides a soft, non-abrasive surface with ample rigidity, thus allowing the birds to be raised on a surface nearly free of feces, yet avoiding many of the animal welfare problems associated with standard cage systems such as breast blisters, folliculitis, and wing and leg breakage/downgrade problems. Beneath the floor of each cage is a belt that collects and dries the manure before it automatically conveyed from the facility. Havenstein et al. (1998) reported that broilers raised in this system have better weight gains and lower mortality rates than those raised on litter floors, and nitrogen volatilization from the manure is greatly reduced thus creating a nutrient-dense by-product that is more easily handled.

The study reported herein was designed to study the effects of grain, particle size, and housing system. It was hypothesized that dietary inclusion of triticale, especially the whole grain, can help maintain intestinal health and discourage enteric pathogen colonization of broilers challenged with *Salmonella*. It was also hypothesized that *Salmonella* colonization in

broilers can be influenced by the type of flooring system (litter versus non-litter) which differ in the degree of bird contact with manure. To test these hypotheses, experimental diets were formulated either with corn or triticale and broilers were raised either in a conventional litter floored house or in the Broilermatic non-litter System. We also studied how the shift in *Salmonella* population by the dietary treatments was related to changes in growth performance, intestinal histomorphometry, gizzard and proventriculus pH.

5.3 MATERIALS AND METHODS

5.3.1 Bird Husbandry

Two-thousand-five-hundred-sixty 1 day-old Ross 508 (Aviagen, Huntsville, AL) broiler chickens obtained from the North Carolina State University hatchery (Raleigh, NC) were weighed, neck-tagged and then orally gavaged with 1 ml (8×10^5 colony forming units – cfu) of a cocktail of *Salmonella enterica* as described below. These birds were then randomly assigned to 2 experimental housing systems: a conventional litter floored 11 m × 30 m curtain-sided house and a 11 m × 24 m power-ventilated house equipped with the Broilermatic non-litter System (Farmer Automatic of America Inc., Register, GA). Forty broilers were randomly assigned to each of the 32 pens used in each house. In the Broilermatic-equipped house the birds were randomly placed into two battery units consisting of two tiers spaced 24 cm apart, with eight pens per tier. Each pen was 120 cm wide, 194 cm long and 42 cm high, providing 582 cm^2 of floor space per bird. Each battery tier was individually equipped with automatic chain feeders and Lubing nipple drinkers. In the conventional house the birds were randomly placed into two blocks of 16 pens, each block separated by a center work area. Each floor pen was 122 cm wide × 366 cm long,

providing 1,116 cm^2 of floor space per bird. Each floor pen was provided with 10 cm of soft pine wood shavings. House temperature and ventilation rate were maintained similarly across the two houses during the experimental period which run from March to April, 2005. House temperatures throughout the trial period included highs of 19° to 39°C (average of 27.5°C) and lows of 14° to 37°C (average of 24.8°C), whereas ambient temperatures included daily highs of 4° to 34°C (average of 18.9°C) and daily lows of -11° to 22°C (average of 6.7°C). Birds were kept on a 24-day light schedule in both houses. Feed and water were given *ad libitum*. Broilers were inspected daily and birds with visual health problems or poor body condition were removed, weighed and euthanized by cervical dislocation. All mortality was weighed so that an appropriated adjustment of feed conversion ratio (FCR) could be made.

5.3.2 Experimental Design and Diets

The experimental design consisted of four dietary treatments each with eight replicate pens of 40 broiler chickens per house. The dietary treatments were randomized within four blocks in each house to account for position effects. Over the entire experimental period (1-42 d), all broilers were fed either a corn- or a triticale-soybean meal (SBM) diet. From 1-14 d of age (starter diet), feed was offered in crumble form and from 15-42 d (grower and finisher diets) in pellet form (Table 1). The experimental diets were formulated without antimicrobials or coccidiostats using least-cost linear programming to meet or exceed the NRC (1994) nutrient requirements. The corn included in the feed was either finely ground or coarsely ground for treatments 1 (C) and 2 (CC), respectively; and the triticale was either finely ground or whole for treatments 3 (T) and 4 (WT), respectively. The corn used to prepare the fine and coarse diets was supplied and ground by Southern States Feed Mill

(Farmville, NC). The finely ground corn had an average particle size of 560 μ while the coarsely ground corn had a final average particle size greater than 3000 μ. The triticale (Tritical-498, Lot No TC-1101-B, Virginia, USA) was supplied by Resource Seeds, Inc. (Golroy, CA) and ground at the North Carolina State University feed mill (Raleigh, NC) using a hammermill (Bliss Industries Inc., Ponca City, OK) equipped with a 3-mm screen, resulting in a final average particle size of 560 μ. The forth experimental diet was prepared using the whole triticale grain. A vertically oscillating sieve shaker (W. S. Tyler, Inc., Mentor, OH) was used to determine particle size distributions of the pelleted diets (grower and finisher) (Grant and Colenbrander, 1990). Approximately 500 g of feed were weighed and placed on the top screen of a stack of 8 sieves (U.S.A. Standard Test Sieve, Fisher Scientific, Bohemia, NY) and then shaken for 20 minutes. The time was predetermined as the point at which the weight of feed particles recovered in the pan did not change. The screen sizes used were 4.75, 3.35, 2.00, 1.70, 1.40, 1.18, 1.00, and 0.425 mm. The arithmetic mean feed particle size and percentage of feed particles recovered on the sieves were calculated.

5.3.3 Enzymes

The triticale-based diets were supplemented with Avizyme 1502 (Danisco Animal Nutrition, Wiltshire, UK), which is a commercial fine granular enzyme preparation obtained from fermentation of *Bacillus subtilis* and genetically modified *Trichoderma longibrachiatum*. The genetically modified *Trichoderma longibrachiatum* produces a heat stable endoxylanase. The enzyme preparation contained standardized activities of at least 600 endo-1,4-beta-xylanase units (EC 3.2.1.8), 8000 units of subtilisin (EC 3.2.1.8) and 800 units of alpha amylase (EC 3.4.21.62) per gram of product.

5.3.4 Bacterial Strains and Inoculum

A cocktail of *Salmonella enterica* subspecies *enterica* serotypes Typhimurium (ATCC 700408), Newport (ATCC 6962), Heidelberg (ATCC 8326), and Kentucky (field isolate) was used as the inoculum. The strain Kentucky had been previously isolated from turkey feces and was serotyped by the NVSL (National Veterinary Service Laboratories, Animal and Plant Health Inspection Services, USDA, Ames, IA). For the preparation of the inoculum, the four strains were grown separately overnight at 37°C in brain-heart infusion (BHI) broth (Oxoid Ltd., Ogdensburg, NY). The cultures were then mixed together and serially diluted in buffered peptone water (BPW, Oxoid Ltd., Ogdensburg, NY) to a final concentration of 8×10^5 cfu/ml. The cell count was determined by direct plating on BHI agar plates and incubation overnight at 37°C. Negative controls were used for all plating procedures to ensure that the media had been properly sterilized.

5.3.5 Serotyping

Salmonella isolates recovered from meconium samples were re-streaked on modified lysine iron agar (MLIA, Oxoid Ltd., Ogdensburg, NY) plates and one isolated colony from each sample was randomly picked, streaked onto tryptic soy agar (Oxoid Ltd., Ogdensburg, NY) slants and shipped to the NVSL for serotype determination. Serotyping was done based on the Kauffmann-White Scheme. For the purpose of this document serovars of *Salmonella enterica* subsp. *enterica* will be referred to as *Salmonella* accompanied by the serotype name. For example, *Salmonella enterica* subsp. *enterica* ser. Typhimurium will be referred to as *Salmonella* Typhimurium (or *S.* Typhimurium).

5.3.6 Data Collection and Analytical Methods

At each sampling time, 1 bird was randomly chosen from each pen, weighed, and euthanized by cervical dislocation. The abdomen was opened and the proventriculus, gizzard, small intestine (duodenum, jejunum and ileum) and ceca were collected. These tissue samples were collected at 7, 14, 21, 28, 35, and 42 days of age; with the exception for the intestinal samples used for histology analysis which were collected on day 3. Feed consumption by pen and individual bird body weight (BW) were recorded at 1, 14, 28, and 42 days of age.

Gizzard and Proventriculus Weights and pH. After being removed from the abdominal cavity, the gizzard and proventriculus were placed separately in Whirl Pak bags (Fisher Scientific, Bohemia, NY) and stored on ice during transport to the laboratory. Upon arrival at the laboratory the contents of the gizzards and proventriculus were separately flushed with sterile deionized (DI) water for pH determination and then the organs were separately weighed. After being flushed with DI water (ca. 1:10 dilution, content:water ratio) into separate sterile Whirl-Pak bags, suspensions were manually homogenized by shaking for 1 minute. A pH probe (Fisher Scientific International, Bohemia, NY) was then inserted directly into each bag and the pH was recorded (Fisher Scientific International, Bohemia, NY). The probe was washed using sterile DI water in between readings.

Salmonella Isolation. Ceca were aseptically removed, weighed, and stored on ice before being quantitatively cultivated for *Salmonella* isolation. Cultivation for *Salmonella* was performed immediately after transport to the lab using the most probable number (MPN) procedure described by Santos et al. (2005) with minor modifications. Briefly, cecal samples were placed in separate 17.78 × 30.48 cm (7 × 12 inch) sterile filtered stomacher bags (Spiral

Biotech Inc., Norwood, MA) followed by the addition of BPW at a 1:10 dilution in each bag. The bags were then homogenized for one minute using a stomacher (IUL Instruments, S.A., Barcelona, Spain). After homogenization, a three tube MPN technique was employed using BPW as a pre-enrichment broth. All tubes were incubated at 37°C for 18 to 24h before transferring 0.1 ml of the appropriate dilutions to triplicate tubes containing 10 ml of Rappaport-Vassiliadis (RV) broth for selective enrichment. All RV broth tubes were incubated at 42°C for 24 h. Following incubation, one loopful from each tube was streaked for isolation onto MLIA and incubated at 37°C for 24 h. Suspect colonies were picked, streaked, and stabbed onto triple sugar iron (TSI, Oxoid Ltd., Ogdensburg, NY) agar slants and then incubated at 37°C for 24 h. Presumptive positive *Salmonella* colonies were confirmed by agglutination using poly-O antiserum (Difco, Sparks, MD). Populations of *Salmonella* for each sample were determined using the Thomas' approximation (Swanson et al., 2001).

At day of placement, meconium samples from all poults were collected and examined for salmonellae using the MPN procedure described above. Meconium samples were collected prior to *Salmonella* challenge. Fecal samples were collected at day 14 and 28 and cultivated for *Salmonella* using the MPN procedure to assure that broilers were colonized by *Salmonella* and actively shedding the pathogen. A composite sample of ten grams of fresh feces was collected beneath each cage and on the litter surface. Twelve hours before fecal collection the belts of the Broilermatic System were cleaned and the old fecal material removed.

Intestinal Hystomorphometry. The small intestine segments (duodenum, jejunum and ileum) and ceca were collected and intestinal weights and lengths measured. The length of

the small intestine was measured for each segment as defined by duodenum: from the gizzard to pancreatic and bile duct, jejunum: from the bile duct to Meckel's diverticulum, ileum: from the Meckel's diverticulum to ileo-cecal-colonic junction, and ceca (Samanya and Yamauchi, 2002). Each segment was placed on a ruler and the length (centimeters) was recorded. Intestinal segment weights (grams) were recorded after its digesta content had been manually removed. Intestinal weights and lengths were calculated relative to bird live body weight (kilograms) (Bjerrum et al., 2005).

For histology analysis, approximately 3 cm of tissue were sampled from the middle part of the jejunum from each bird at 3 days of age (Salgado et al., 2002; Samanya and Yamauchi, 2002). The lumen content of each 3 cm jejunum section was washed by injecting a solution of 10% formalin fixative buffer (Salgado et al., 2002; Samanya and Yamauchi, 2002). Immediately, the jejunum section was placed into clean 10% fixative formalin buffer solution for 24 hours. The fixed jejunum segment was then processed according to a modification of the methods described by Iji et al. (2001) and Samanya and Yamauchi (2002). Briefly, one 3 mm section was taken from each jejunum segment of each bird. A total of 8 chicks were randomly chosen per dietary treatment. The section was enclosed in tissue cassettes and placed in 75% ethyl alcohol solution until processed by the histology laboratory (Histopathology Laboratory, College of Veterinary Medicine, NCSU, Raleigh, NC). The fixed jejunum sections enclosed in the tissue cassettes were embedded in paraffin wax within 72 h to avoid artifacts. Transverse sections of 5-µm thick were cut with a rotary microtome and stained with Lilee Meyer haematoxylin and counter-stained with eosin yellow. The transverse section slides were digitized using a Micromaster I microscope (Fisher Scientific International, Bohemia, NY) and the images were analyzed using Image Tool software

(UTHSCSA Image Tool Software, V. 3, The University of Texas, San Antonio, TX). Images were viewed to measure villus height, villus apical width at the villus tip, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth as described by Iji et al. (2001). Apparent villus surface area was estimated from the trigonometric relationship between villus height, villus basal width and villus apical width (Iji et al., 2001). Mucosal height was calculated from crypt depth plus villi height measurements. Villus height to crypt depth ratio was also calculated. Ten individual villi were assessed per section. An average of the 10 measurements assessed per section was expressed as a mean for the corresponding jejunum segment. Each bird was the experimental unit for statistical analysis.

Carcass Yield. On day 42 of the trial, 2 birds per pen and cage (16 birds per treatment per house) were selected at random for estimation of carcass characteristics. Feed was removed for 12 h before processing. Birds were allowed access to water during the first four hours of the feed withdrawal period. At the time of slaughter, the fasted BW (live weight) of each bird was measured immediately prior to stunning and exsanguination. All birds were electrically stunned, killed by hand using a conventional unilateral neck cut to sever the carotid artery and jugular vein, bled for 180 s, scalded at 63°C for approximately 120 s, and were then placed into a rotary drum mechanical picker for 30 s.

After the head, shanks and feet were removed, carcasses were eviscerated by cutting around the vent to remove the abdominal fat pad and all of the viscera except the kidneys. Once eviscerated, carcasses without giblets were weighed to determine the eviscerated carcass weight (hot dressed yield) (Havenstein et. al., 2003). The weights of the heart, liver, gizzard, total intestines (small and large) and abdominal fat pad were also weighed. The hot carcasses were then submerged overnight in an ice-water bath without agitation. After

overnight chilling the carcasses were then drained and reweighed to calculate water uptake (iced carcass weight). Each carcass was then cut into its component parts. Further processing was completed for all birds within approximately 1 h from the time the birds were removed from the ice bath. After reweighing of the drained carcasses, each was placed on a cone and cut into its component parts: wings, drumsticks, thighs, breast skin, pectoralis major, pectoralis minor, and rack (i.e., the thoracic vertebrae and ribs with overlying skin and muscle, the clavicle, the sternum, and neck) along with the back posterior (total rack weight). All weights were recorded to the nearest 0.01 g.

5.3.7 Statistical Analysis

Statistical analysis of results was performed using the general linear model procedure (GLM) of SAS (SAS Institute, 1996) according to the following general model: $Y_{ijklm} = \mu + \alpha_i + \beta_{j(l)} + \delta_k + \gamma_l + (\alpha\delta)_{ik} + (\alpha\gamma)_{il} + (\delta\gamma)_{kl} + E_{ijklm}$, where Y_{ijklm} was the observed dependent variable; μ the overall mean; α_i the age effect; β_j the block effect which is nested in the house effect; δ_k the dietary treatment effect; γ_l the house effect; $(\alpha\delta)_{ik}$ the interaction between age and dietary treatment; $(\alpha\gamma)_{il}$ the interaction between age and house; $(\delta\gamma)_{kl}$ the interaction between dietary treatment and house; and E_{ijklm} the random error. Replicate pens or cages of 40 birds each served as experimental units. When treatments effects were found to be significant using the F-test ($P < 0.05$), the treatment means were separated by the least-square-means (lsmeans) function of SAS (SAS Institute, 1996) with a confidence of $P < 0.05$. Before statistical analysis, all MPN data were transformed to the base-10 logarithm and the intestinal measurement data were transformed relative to body weights. Finally, correlations between *Salmonella* cecal population, relative gizzard, proventriculus and ceca weights, and

gizzard and proventriculus pH were accomplished using the correlation procedure of SAS and the results of the correlation analysis were expressed as r values.

5.3.8 Animal Ethics

The experiments reported herein were conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were performed with full consideration of animal welfare.

5.4 RESULTS

5.4.1 Diet Particle Size and Performance

The arithmetic mean particle size of the four dietary treatments were 3.39, 3.69, 4.25, 2.99 mm for treatments 1 (C), 2 (CC), 3 (T) and 4 (WT), respectively (Table 2). The overall average recovery of the four dietary treatment samples from the screen sieves was 99.86%. The most noticeable differences between the particle size distributions were the percentage of particles retained on the first three screens (4.75, 3.35, 2.00 mm). Finely ground triticale (T) produced the best quality pellet of the four treatments, whereas whole triticale was the worst ($P < 0.01$).

Grain type and particle size effects on body weight, body weight gain (BG) and feed consumption (FC) were dependent upon housing design (Tables 3-5). Feeding finely ground grain resulted in greater BW, BG and FC than feeding coarsely ground or whole grain ($P < 0.05$), yet no differences in FCR were observed in birds reared on litter. Conversely, only grain type affected performance of broilers raised in the Broilermatic cages. Dietary

inclusion of triticale, especially finely ground, increased ($P < 0.01$) BW of birds raised in cages, with no statistical differences in FC and FCR detected in comparison to the corn-SBM control treatment.

Generally, broilers reared on the litter were heavier than those reared in cages (1.40 vs. 1.33 kg, $P = 0.04$). Although broilers raised on litter and fed finely ground grain gained about 315 g more weight over 42 days than birds fed coarsely ground or whole grain, they also consumed approximately 8% more feed ($P = 0.001$). Therefore, there was no statistical difference in the interaction between house and dietary treatments for periodic FCR; but, a significant house effect was detected (Table 6). Although the cage-reared broilers had a better FCR than the litter-reared ones from 1 to 14 days (1.18 vs. 1.22 g/g, $P = 0.01$), by the end of the experiment (42 d) the litter-reared birds had about 6% lower cumulative FCR than the cage-reared birds (1.71 vs. 1.81 g/g, $P = 0.02$). The overall mortality rate of 12.8% was within industry standards and was not significantly influenced by housing design, grain type or particle size.

5.4.2 Gizzard and Proventriculus

Gizzard weight was influenced by housing design and dietary treatments, whereas proventriculus relative weight was affected only by the diets (Table 7). There were no housing × diet interaction effects observed for the gizzard and proventriculus relative weights. On average, the relative gizzard weight of broilers raised on litter was significantly higher than those in cages (22.2 vs. 20.8 g/kg, $P < 0.0001$). Regardless of grain type, broilers fed coarser diets had heavier gizzards than those fed the fine ground grain diets (23.42 vs. 19.56 g/kg, $P < 0.0001$). In contrast, proventriculus weights were statistically different

among the dietary treatments (Table 7). Contrast analysis revealed that birds fed triticale-based diets had a heavier proventriculus than corn-fed broilers (5.9 vs. 6.3 g/kg, $P = 0.0004$).

Gizzard and proventriculus pH were significantly influenced by housing and dietary treatments (Table 8). The gizzard contents of broiler raised on litter were generally more acidic than those reared in cages at 14 d (3.1 vs. 3.3, $P = 0.04$), 21 d (3.8 vs. 4.2, $P < 0.0001$), 28 d (4.2 vs. 4.9, $P < 0.0001$) and 35 d (4.4 vs. 4.7, $P = 0.03$). In contrast, housing systems inconsistently affected the pH of the proventriculus contents across age categories; the proventriculus contents were less acid among cage-reared birds at 21 d, but more acid at 28 d ($P < 0.0001$) and at 35 and 42 d, housing had no significant effect on proventriculus pH.

The gizzard contents of broiler fed coarse ground or whole grain were generally more acidic than those feed finely ground grain at 21 d (3.9 vs. 4.1, P 0.0006), 28 d (4.3 vs. 4.8, $P < 0.0001$), 35 d (4.4 vs. 4.7, $P = 0.01$) and 42 d (4.2 vs. 4.5, $P < 0.01$). Conversely, the proventriculus contents of broilers fed triticale (either finely ground or whole) were more acidic than the contents of birds fed corn-based diets throughout the entire course of the experiment (4.1 vs. 4.3, 14 d; 3.9 vs. 4.1, 21 d; 4.3 vs. 4.5, 28 d; 4.7 vs. 5.2, 35 d; and 4.9 vs. 5.2, 42 d; $P \leq 0.01$).

Significant correlations were observed between the size of the gastric organs and the pH of their contents (Table 8). The relative weights of the gizzard and proventriculus were positively correlated ($r = 0.37$, $P< 0.0001$) as were the pH of the gizzard and proventriculus contents ($r = 0.87$, $P < 0.0001$). In addition, the relative weight of the gizzard was inversely correlated with the pH of its contents ($r = -0.62$, $P < 0.0001$), as was the relative weight of the proventriculus and the pH of its contents ($r = -0.29$, $P < 0.0001$).

5.4.3 Intestinal Tract

Relative small intestine weights and lengths are summarized in Tables 9 through 11. The relative weight and length of the small intestine were significantly ($P < 0.01$) less in broilers raised on litter than in those raised in cages. At 42 d, the litter-raised broilers had significantly lower relative weights and lengths of jejunum (9.69 vs. 10.28 g/kg, $P < 0.10$; 20.6 vs. 22.8 cm/kg, $P = 0.02$) and ileum (7.41 vs. 8.68 g/kg, $P = 0.001$; 20.4 vs. 23.1 cm/kg, $P = 0.002$). Similarly, the duodenum length of litter-reared broilers was 7% less than the cage-reared birds at 28 d, (16.69 vs. 17.79 cm/kg, $P = 0.04$).

The dietary treatments had no significant effect on relative small intestine weights. However, the diets significantly influenced relative jejunum and ileum length at 14 and 42 d, respectively. Feeding whole triticale or fine ground corn decreased the relative jejunum length when compared to feeding fine ground triticale or coarse ground corn at 14 d (89.8, 90.0, 104.9, 104.3 cm/kg, respectively, $P = 0.01$). However at 42 d, the corn-based diets yielded increased relative ileum length by 11% as compared to the triticale-based diets (22.9 vs. 20.6 cm/kg, $P < 0.005$).

In contrast to what was observed with the small intestine, the relative ceca weight was influenced by housing design and not by diet (Table 12). The ceca weights of broilers raised on litter were significantly lower than those raised in cages at 28 d (4.36 vs. 5.36 g/kg, $P < 0.0001$) and 42 d (3.74 vs. 4.42 g/kg, $P = 0.01$). The only detected dietary treatment effect on ceca measurements was in the cage-reared birds at 14 d of age; ceca length was significantly lower in birds fed the fine ground corn diet than those fed other diets.

Histological measurements of the jejunum was minimally affected by dietary treatment, but was impacted by housing type (Table 13). Contrast analysis revealed that

only the villus apical width increased when broilers were fed triticale (58.96 vs. 54.36, P = 0.05). Villus height, villus area, villus-height to crypt-depth ratio and mucosal depth were significantly larger for broilers reared on litter, regardless of dietary treatment (P < 0.01). There was no significant difference in villus apical and basal width, crypt depth and muscularis depth between the birds raised on litter and those raised in cages.

5.4.4 Carcass Yield

Dietary treatments and housing design significantly (P < 0.05) influenced carcass yields of broilers at 42 days of age (Table 14). In comparison to the litter-reared broilers, the cage-reared birds had significantly lower liver, gizzard and abdominal fat relative weights and increased thigh meat relative weight. Regardless of housing, the abdominal fat relative weight was significantly (P < 0.01) greater in broilers fed the corn-based diets than those fed triticale-based diets. In comparison to the finely ground diets, the diets containing the coarse ground corn or whole triticale increased the relative gizzard weight by 45% yet decreased the relative eviscerated carcass weight (752.27 vs. 761.92 g/kg, P < 0.01).

Depending on housing design, dietary treatments influenced relative breast weight and total meat yield (Table 15). Contrast analysis showed that raising broilers on litter and feeding finely ground grain diets, regardless of grain type, increased the relative breast weight (214.53 vs. 202.69 g/kg, P = 0.02) and meat yield (525.50 vs. 511.77 g/kg, P = 0.056) when compared to broilers fed whole or coarsely ground grain diets. In contrast, raising broilers in cages and feeding triticale-based diets, regardless of grain particle size, increased the relative breast weight (210.80 vs. 193.76 g/kg, P < 0.001) and meat yield (532.58 vs. 510.03 g/kg, P = 0.01) compared to broilers fed corn-based diets.

5.4.5 Salmonella Cecal Colonization and Fecal Shedding

MPN and serotyping analyses of meconium samples showed that chicks were naturally infected with *Salmonella* Infantis. However, there were no significant ($P > 0.05$) differences in *Salmonella* populations recovered from the meconium samples, which averaged 2.38 log MPN/g (Table 17). Additionally, cecal *Salmonella* populations at 7 d were not affected by dietary treatment (Figure 1) or housing type (Figure 2), confirming that all birds started with an equivalent dose of *Salmonella*.

Salmonella cecal colonization was influenced by housing design (Figure 1) and dietary treatments (Figure 2), whereas *Salmonella* fecal shedding was only influenced by housing design (Table 17). Feeding triticale-based diets resulted in lower *Salmonella* populations in the ceca of 14 d broilers than feeding the corn-based diet (7.33, 7.21, 6.51, 6.61 log MPN/g for C, CC, T and WT, respectively, $P > 0.01$; Figure 1). Grain type and grain particle size influenced *Salmonella* colonization at 28 d. The triticale-based diets resulted in lower *Salmonella* cecal populations than the corn-based diets (5.64 vs. 6.16 log MPN/g, $P = 0.02$). Coarse ground corn or whole triticale resulted in lower cecal *Salmonella* populations than the finely ground diets at 28 d (5.57 vs. 6.23 log MPN/g, $P = 0.002$) and 42 d (3.85 vs. 4.40 log MPN/g, $P = 0.01$).

The litter-reared broilers had significantly lower cecal *Salmonella* populations than the cage-reared birds at 28 d (5.53 vs. 6.27 log MPN/g, $P < 0.01$), 35 d (4.13 vs. 5.43 log MPN/g, $P < 0.01$), and 42 (3.81 vs. 4.44 log MPN/g, $P < 0.01$) (Figure 2). Similarly, *Salmonella* fecal shedding was significantly ($P < 0.01$) lower in litter-reared broilers than broilers reared in cages (Table 17). Correlation analysis revealed no significant ($P > 0.05$) correlation between gizzard pH or proventriculus pH and *Salmonella* cecal populations.

However, there were small but positive correlations detected between relative ceca weights and *Salmonella* populations ($r = 0.30$, $P < 0.05$) and ceca lengths and *Salmonella* populations ($r = 0.12$, $P < 0.05$) (Table 16).

5.5 DISCUSSION

Several differences in growth performance and pathogen colonization were observed between broilers reared in the Broilermatic litter-free System and those raised on litter. Moreover, these differences were in some cases significantly influenced by the type of grain in the diet and the particle size of that grain. Broilers raised on litter had better performance when fed finely ground grain, regardless of grain type. This improvement in growth rate by feeding diets containing finely ground grain is most likely associated with the improved pellet quality which enhances feed consumption. Engberg et al. (2002) and Bennett et al. (2002b) demonstrated increased feed intake and body weight gains by feeding pellets. Supplementing pelleted diets with whole wheat and whole barley decreased the growth rate of chickens (Bennett et al., 2002b), similar to what was found in the present study. Conversely, the influence of grain type (corn vs. triticale) on growth performance of broilers raised in cages was much greater than that of grain particle size. Regardless of particle size, triticale-based diets increased body weight gains yet had no effect on feed consumption or feed conversion ratios. Other published studies have shown that increased addition of high fiber cereals in broiler (Plavnik et al., 2002) and turkey (Santos, 2006) diets improved weight gain and feed conversion ratios.

The major difference between the Broilermatic System and the conventional litter floored house was access to litter. In the conventional house broilers were grown on litter

having free access to the pine shavings whereas the only source of fiber for broilers raised in the Broilermatic system was from the non-starch polysaccharides (NSP) present in the diets. Cereal non-starch polysaccharides, major components of dietary fiber, are resistant to hydrolysis by digestive enzymes (Trowell et al., 1976). The difference in total NSP between the corn-based diets and triticale-based diets was on average 40 g/kg. Also, triticale-based diet had about 33% more soluble-NSP than the corn-based diets. This data suggest that there may be a minimum fiber intake requirement for birds. Studies with humans (Marlett et al., 2002), broilers (Jimenez-Moreno et al., 2004, 2006) and many other animals (NRC, 1995) have shown the importance of a minimum daily intake of fiber for normal gastrointestinal function. Thus, the corn-based diets may not have provided sufficient fiber required to sustain normal intestinal development and function. However, the access to litter likely fulfilled the fiber requirements of the birds although the actual amount of litter consumed was not measured in the present study. This conclusion is supported by the observation that the litter-reared broilers had significantly greater jejunum villus height, villus area, villus-height to crypt-depth ratio and mucosal depth than cage-reared birds, regardless of dietary treatment. Furthermore, the FCR was significantly lower by about 5% when broilers were reared on litter, confirming that access to litter material improved the efficiency of nutrient uptake.

The experimental diets had no significant effect on the total size of the small intestine. However, litter-raised broilers had lighter and shorter relative small intestines than cage-reared broilers. The difference was attributed mostly to reduced ileum and jejunum weights and lengths. A possible reason to this finding is that broilers raised on litter had reduced small intestine mass that may have improved microbial stability in the intestinal tract

and lower pathogen load. Humphrey et al. (2002) reported that germ-free chickens had lower intestinal mass than conventional chickens. Also, antibiotic-fed chicks have lower small intestine mass than chicks fed antibiotic-free diets (Coates et al., 1955). Sonnenburg et al. (2004) reported that a healthy intestine is composed of protective dense communities of commensal microorganisms that are permanent residents of the intestinal tract. They concluded that an increased microbial diversity would result in the development of stable microbial communities and decreased intestinal mass, while maintaining intestinal efficiency. Intestinal microbial community diversity is recognized as an important host defense mechanism against infection (Abrams and Bishop, 1966). Accordingly, we observed that broilers raised on litter had lower *Salmonella* populations than birds raised in cages.

Generally, the small intestine weight of broilers was lower when the birds consumed higher amounts of fiber, either from the triticale-based diets, free access to the litter, or both. Apparently, the absence of dietary fiber was compensated by an enlargement of the intestine to increase absorptive area. The histological analysis of the jejunum demonstrated that the cage-reared broilers had reduced mucosal depth (due to shorter villus) as compared to the litter-reared birds. Decreased villus area and villus height/crypt depth ratio (an indicator of impaired epithelial cell proliferation, Pluske et al., 1997) were also observed in the cage-reared broilers. In contrast to the cage-reard birds, the litter-reared birds had healthier jejunums with greater absorptive area for nutrient uptake. In conclusion, broilers consuming adequate fiber from the diet or litter will support favorable growth performance characteristics.

Similar to the observations seen for the small intestine, the relative ceca weights were mainly influenced by housing design; litter-reared broilers had lower ceca mass than cage-

reared birds. The ceca are the primary fermentation chambers of the avian gut, where they play an important role in the microbial degradation of some carbohydrates, absorb water and support microbial synthesis of vitamins and degradation of nitrogenous compounds (Józefiak et al., 2004). The size of the ceca is directly related to the degree of fermentation that occurs in the organ. The most developed avian ceca are found in granivores and species of birds whose diets contain elevated levels of plant fiber or chitin (Stevens and Hume, 1998). Therefore, it was expected that litter-reared broilers would have greater cecal mass than the cage-reared birds. However, the opposite was observed possibly because the overall intestinal tract of broilers reared in cages was enlarged as discussed previously.

Gizzard and proventriculus pH were also influenced by housing systems and dietary treatments. In general, gizzard pH was more acidic when broilers were reared on litter, probably related to the extra fiber intake due to litter consumption. Although the proventriculus pH of young birds were influenced by housing design, the impact of dietary treatments on proventriculus pH was much greater than was the influence of housing systems. Feeding whole grain and triticale-based diets to broilers resulted in a more acid gizzard and proventriculus environment, respectively. Previous studies also showed a significant reduction in the gizzard pH in response to whole wheat feeding (Engberg et al., 2004; Bjerrum et al., 2005). As the authors reported, the low pH was caused either by stimulation of bacterial fermentation or an increased secretion of hydrochloric acid by the proventriculus, or both. Results of the present study support some of these findings. There was a significant inverse correlation between the size of the gizzard and the pH of its contents. A similar correlation was also detected with the size of the proventriculus and the

pH of its contents. Evidently the digesta acidity increases as the size of the gastric organs increase.

Feeding broilers coarser diets significantly increased the relative gizzard weight throughout the trial (7 to 42 d), similar to the findings of Engberg et al. (2004) and the previous experiment (Chapter 4). The increased gizzard weight was associated with the increased mechanical stimulation in response to the ingestion the coarse feed particles. Engberg et al. (2002) observed an increased luminal content in the upper small intestine (duodenum and jejunum) after birds were fed pelleted feed. The authors suggested that pellets made with finely ground grain readily disintegrate in the crop and esophagus and pass directly through the proventriculus and gizzard into the duodenum. It has also been reported that the stimulation of gizzard activity by coarsely ground grain or whole grain may modulate pancreatic enzyme activity in the duodenum, thereby regulating the transit of starch by avoiding starch overload in the small intestine and improving starch digestibility (Svihus and Hetland, 2001). Access to litter material and thus higher insoluble fiber also increased relative gizzard weights by stimulating the organ. Increased relative gizzard weights were observed after supplementing corn-based turkey diets with 4% pine shavings (Chapter 4). Similar results were reported in broilers fed diets supplemented with oat hulls (Hetland and Svihus, 2001). The relative weight of the proventriculus was only influenced by the dietary treatments. Independent of housing design, broilers that consumed the triticale-based diets had a heavier proventriculus than those fed corn-based diets. Apparently the impact of higher soluble fiber content (higher in triticale diets) on proventriculus development was much greater than that of the insoluble fiber coming from the litter.

The observed carcass yield data were in accordance with the performance results. Relative gizzard weights of the broilers processed at 42 d were similar to the relative gizzard weights observed from birds sampled throughout the study (7 to 42 d). Relative gizzard weights of processed birds were greater among broilers that were either reared on litter or fed the coarse diets. Broilers raised on litter also had heavier livers, lighter thighs and more abdominal fat than those raised in the litter-free system. In general, birds fed finely ground grain produced heavier eviscerated carcasses than those fed coarser diets. However, the yields of white meat and total meat followed a response trend similar to body weight. Feeding finely ground grain in the litter house and feeding triticale-based diets in the Broilermatic System resulted in greater white and total meat yields. In comparison, Bennett et al. (2002b) observed that carcass yield of broilers were not adversely impacted by feeding whole wheat or barley. The authors also reported no significant difference in eviscerated carcass weights between diets containing ground and whole wheat and barley.

The experimental diets evaluated in this study were formulated as a practical means to test different levels of dietary fiber and grain particle sizes on broiler performance, GIT development and *Salmonella* populations. The comparison of the corn- and triticale-based diets allowed for the evaluation of soluble NSP in the diet, whereas grain structure was evaluated by comparing the impact of feeding coarse and finely ground corn and whole triticale. This experimental design allowed us to test the hypothesis: *Salmonella enterica* colonization decreases as grain particle size and dietary NSP level increase. The soluble NSP are primary substrates for the fermentation of important commensal bacteria found in the GIT (i.e. *Lactobacillus* and *Bifidobacterium*), which can competitively prevent the colonization of enteric pathogens (Lan, 2004). Soluble-NSP are often completely degraded,

easily digestible and more fully fermented by the GIT microflora than insoluble-NSP (Högberg, 2003). To enhance the impact of dietary NSP, the triticale-based diets were supplemented with 600 EXU of endoxylanase per kg of feed. Previous studies have shown that enzyme supplementation of wheat- or triticale-based diets decreased *Salmonella* cecal colonization in turkeys (Santos, 2006). Appropriate enzyme supplementation to cereal-based diets were shown to decrease intestinal viscosity and increase performance and nutrient digestibility of broilers (Flores et al., 1994; Silva and Smithard, 2002) and laying hens (Coon et al., 1988; Lázaro et al., 2003). Cereals, such as wheat and triticale, can replace corn in diets and maintain broiler growth performance if sufficient pentosanase activity is supplemented in the diet (Flores et al., 1994; Brum et al., 2000). Additionally, enzyme supplementation to diets high in NSP content can promote the development of a more healthy enteric microflora ecosystem (Steenfeldt et al., 1998).

As hypothesized, *Salmonella* colonization of broiler intestine was discouraged by a diet high in NSP content. At 14 d, broilers fed the corn-based diets were more heavily colonized than the triticale-fed birds (ca. 1 log MPN/g) difference. However, the influence of grain particle size on *Salmonella* colonization took longer to develop than the dietary fiber content. Broilers fed the coarse diets had lower *Salmonella* cecal populations by around 1 and 0.5 log MPN/g at 28 and 42 d, respectively. Similar responses were observed by other investigators. Bjerrum et al. (2005) showed that whole wheat feeding decreased *Salmonella* Typhimurium populations in the ileum of broilers. Engberg et al. (2004) observed a reduction in the population of lactose-negative enterobacteria (several potentially pathogenic bacteria are members of this group including *Salmonella*) throughout the GIT with the exception of the ceca. *Salmonella* was more effectively reduced in our study due to the inclusion of

higher concentrations of coarser particles in the CC and WT experimental diets. The corn used in the CC diet was exclusively coarsely ground and the triticale was added exclusively as whole grain in the WT diet.

Broilers fed the triticale-based diets contained fewer *Salmonella* organisms than those fed the corn-based diets. *Salmonella* populations at 28 d were higher in broilers fed the corn-based diets than those fed the triticale-based diets; but by 42 d, there was no statistical difference due to grain type among dietary treatments. Although all treatments had similar *Salmonella* populations at 7 days, *Salmonella* populations increased by 0.1 log MPN/g from 7 to 14 d in birds fed the corn-based diets yet decreased by 0.6 log MPN/g in those fed the triticale-based diets. These findings agree with Santos (2006) who reported a faster rate of reduction in *Salmonella* populations among turkey toms fed wheat- and triticale-based diets than those fed corn-based diets. The author demonstrated that toms were more capable of recovering from a *Salmonella* infection when fed wheat- or triticale-based diets.

Cecal *Salmonella* populations and fecal shedding of *Salmonella* were significantly influenced by housing design. *Salmonella* populations were 0.6 log MPN/g higher in fecal samples collected from 14 d broilers reared in cages than from birds reared on litter. By 28 d, *Salmonella* cecal and fecal populations among cage-reared broilers were higher than litter-reared birds by 0.7 and 1.1 log MPN/g, respectively. The difference between the two housing systems reached a maximum of 1.3 log MPN/g at 35 d for the cage-reared birds over those reared on litter. By 42 d the difference between these two systems had declined to 0.6 log MPN/g. Considering that the broilers raised in both housing facilities were fed the same diets, the difference between the two is presumably related to the access to litter. Contrary to the general assumption that litter consumption increases *Salmonella* colonization through

reinfection, the present study showed a consistent reduction in *Salmonella* populations for birds reared on litter. Presumably the coarse wood components in litter plays a major role in reducing pathogen colonization in the avian ceca, perhaps by the mechanical stimulation of the gizzard and proventriculus or by serving as a seeding agent for competitive exclusion microorganisms.

Dietary NSP, including the high NSP content present in litter, is believed to have beneficial effects on the gastrointestinal tract of poultry. These benefits include the shift of microbial populations towards one that competitively excludes harmful microorganisms that disrupt the host-microflora ecosystem. Dietary NSP are important substrates for these commensal enteric bacteria that by utilizing available nutrients, occupy attachment sights on the brush border epithelial cells and maintain a habitat unfavorable to pathogens. Additionally, some NSP can directly or indirectly enhance natural immunocompetence of poultry (Zhang et al., 2004; Kataoka et al., 2002). Arabinoxylan, which is a major NSP present in triticale, has been shown to directly activate a macrophage cell line in the broiler intestine thus conferring a protective effect against pathogen colonization (Zhang et al., 2004). Similarly, β-glucan, another NSP found in triticale, increases mammalian macrophages and neutrophils concentration in cell cultures (Kataoka et al., 2002) and confers a protective barrier against *Salmonella* in broiler chickens by up-regulating heterophil phagocytosis, bactericidal killing and oxidative burst (Lowry et al., 2005). Lastly, dietary NSP indirectly stimulate the immune system by encouraging the growth of enteric lactic acid bacteria which through their cell wall components (Takahashi et al., 1993; Haller et al., 1999) evoke an immune response (Stewart-Tull, 1980), especially on the intestinal mucosal surface (Link-Amster et al., 1994). Therefore, feed ingredients containing high levels of NSP and

access to litter would both serve as potential prebiotic sources that could promote intestinal health by encouraging the proliferation of commensal microflora and discouraging the colonization of *Salmonella* species in the intestinal tract of broilers.

In conclusion, the results of this study showed that dietary NSP content, housing design, grain type and particle size impact intestine size and histomorphometry as the bird adapts to changes in nutrient digestibility. This study also demonstrated that replacing corn with triticale in broiler diets and using coarsely ground or whole grain can have a significant effect on *Salmonella* colonization and intestinal health of broilers. Furthermore, dietary inclusion of coarse grain particles or a grain high in dietary fiber content, such as triticale, can discourage *Salmonella* colonization in broiler ceca. Finally, rearing broilers in a conventional litter-based house was superior to a non-litter cage-based housing system in terms of growth performance and resistance to colonization following a *Salmonella* challenge.

ACKNOWLEDGMENTS

This study was supported by the USDA Initiative for Future Agriculture and Food Systems (IFAFS) grant. The authors wish to thank Annette Israel, Jamie Warner, Jean de Oliveira, Ondulla Foye, Renee Plunske, Mike Mann, Robert Neely and the NCSU Poultry Educational Unit farm employees, Raleigh, NC, for their technical assistance during this study. Appreciation is also extended to Southern States Feed Mill (Farmville, NC) for providing and grinding the corn used in the experimental diets, and to Resource Seeds Inc. (Golroy, CA) for providing the triticale used in the experimental diets.

5.6 TABLE AND FIGURES

Table 1. Composition and nutrient content of the experimental diets containing different particle sizes of corn and triticale fed to broilers from 1 to 42 days

INGREDIENTS (%)	Starter (1 – 14 d)		Grower (15 – 28 d)		Finisher (29 – 42)	
	Corn	Triticale	Corn	Triticale	Corn	Triticale
Corn, Grain	59.90	0.00	66.78	0.00	73.27	0.00
Triticale	0.00	58.04	0.00	64.71	0.00	70.97
SBM (48% CP)	27.40	27.80	22.20	22.60	16.90	17.40
Poultry Meal (60% CP)	5.00	5.00	5.0	5.00	5.0	5.0
Poultry Oil	3.90	5.46	2.86	4.62	2.00	3.91
Limestone	1.20	1.17	1.23	1.21	1.13	1.10
Dicalcium Phosphate	1.43	1.34	0.90	0.81	0.65	0.55
Salt	0.40	0.34	0.36	0.30	0.37	0.31
Vitamin Premix[1]	0.10	0.10	0.10	0.10	0.10	0.10
Mineral Premix[2]	0.20	0.20	0.20	0.20	0.20	0.20
Choline Chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.18	0.20	0.08	0.10	0.09	0.11
L-Lysine HCl	0.04	0.00	0.04	0.00	0.04	0.00
Selenium Premix[3]	0.05	0.05	0.05	0.05	0.05	0.05
Avizyme 1502	0.00	0.10	0.00	0.10	0.00	0.10
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00
			Calculated Analysis			
ME, kcal/kg	3200	3200	3200	3200	3200	3200
Protein, %	22.0	22.0	20.0	20.0	18.0	18.0
Calcium, %	1.0	1.0	0.90	0.90	0.80	0.80
Available Phosphorus, %	0.45	0.45	0.35	0.35	0.30	0.30
Sodium, %	0.22	0.22	0.20	0.20	0.20	0.20
Lysine, %	1.20	1.20	1.05	1.06	0.90	0.90
Methionine+Cysteine, %	0.95	0.95	0.80	0.80	0.75	0.75
			Chemical Analysis[4]			
Dry Matter, %	89.75	90.96	89.30	90.02	89.45	90.33
Crude Protein, %	20.19	22.94	21.88	20.88	20.69	21.75
Gross Energy, kcal/kg	4461.26	4531.37	4484.17	4555.76	4397.17	4466.15
Fat, %	7.10	8.07	6.82	7.66	6.04	6.28
Ash, %	5.68	5.59	4.75	5.53	5.66	4.27
Fiber Total, %	12.87	18.40	22.54	23.09	11.79	17.71
Insoluble, %	5.17	7.43	10.00	9.80	4.44	7.07
Soluble, %	7.70	10.97	12.54	13.30	7.36	10.64

[1] Supplied the following per kilogram of feed: vitamin A, 26,400 IU; cholecalciferol, 8,000 IU; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4 mg; pyridoxine, 15.8 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 8 mg; biotin, 0.506 mg; vitamin B12, 0.08 mg; ethoxyquin, 200 mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate.

[2] Supplied the following per kilogram of feed: 120 mg Zn as $ZnSO_4 \cdot H_2O$; 120 mg MN as $MnSO_4 \cdot H_2O$; 80 mg Fe as $FeSO_4 \cdot H_2O$; 10 mg Cu as $CuSO_4$; 2.5 mg I as $Ca(IO_3)_2$; 1.0 mg Co as $CoSO_4$.

Table 1. Continued

[3] Selenium premix provided 0.3 ppm Se from sodium selenate.

[4] Chemical analysis: Crude protein used Kjeldahl automatic analyzer (Kjeltec Auto 1030 Analyser, Tecator, Sweden). Gross energy used bomb calorimeter (IKA Calorimeter System C5000 control, IKA Werke Labortechnik, Staufen, Germany). Fat used ether extract (Labconco Corporation, Kansas City, MO) method. Ash used muffle oven (Thermolyne, Sybron Corporation, Dubuque, IA) method. Dietary fiber analysis was performed by Intertek Agri Services Food Agricultural Testing Laboratory (St. Rose, LA) using the AOAC International standard method for soluble, insoluble and total dietary fiber analysis.

Table 2. Particle size distribution of the experimental diets (dry sieve method)

	DIETARY TREATMENTS					
ITEM	**C[2]**	**CC[3]**	**T[4]**	**WT[5]**	**P-value**	**SEM(8)**
Particle Size[1] (mm)	3.39	3.69	4.25	2.99	-	-
Screen Size (mm)	**(% Retained)[6]**					
4.75	53.00^{B}	58.15^{B}	84.27^{A}	30.48^{C}	0.0003	5.4755
3.35	11.35^{C}	16.39^{B}	2.58^{D}	21.28^{A}	< 0.0001	1.2448
2.00	15.23^{B}	14.12^{B}	4.02^{C}	33.03^{A}	< 0.0001	2.6147
1.70	3.44^{A}	1.62^{B}	1.12^{C}	4.25^{A}	0.0018	0.5051
1.40	4.33	2.47	2.11	3.75	0.2258	0.8509
1.18	1.64^{a}	0.81^{b}	0.56^{b}	1.00^{ab}	0.0344	0.2133
1.00	1.39	0.82	0.67	0.95	0.2871	0.2708
0.43	6.48	3.62	3.40	4.17	0.5170	1.6312
Pan	2.87	1.90	1.14	1.02	0.4041	0.7373
TOTAL	**99.73**	**99.91**	**99.88**	**99.93**	-	-

[1] Arithmetic mean particle size of 4 composite samples of pelleted dietary treatments (2 composite grower and 2 composite finisher feed samples/treatment).
[2] C: finely ground corn.
[3] CC: coarsely ground corn.
[4] T: finely ground triticale.
[5] WT: whole triticale.
[6] Particle size distribution obtained from an oscillating shaker.
[a,b] Means with different superscripts within a row differ significantly ($P < 0.05$).
[A,B,C,D] Means with different superscripts within a row differ significantly ($P < 0.01$).

Table 3. Effect of housing design, grain type and particle size on body weight[1] of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	14 d	28 d	42 d
Litter House		**(g)**	
C[2]	405.2^{A}	1455.2	2858.9^{A}
CC[3]	362.1^{C}	1328.7	2685.5^{B}
T[4]	392.0^{AB}	1443.2	2867.9^{A}
WT[5]	369.6^{BC}	1369.9	2726.1^{B}
P-value	0.0050	0.0484	0.0010
SEM(25)[6]	8.5058	34.6646	33.95
Broilermatic		**(g)**	
C	349.4^{b}	1315.5	2606.3^{b}
CC	360.5^{ab}	1326.1	2647.0^{b}
T	354.5^{b}	1354.1	2752.9^{a}
WT	366.6^{a}	1325.4	2671.2^{ab}
P-value	0.0392	0.4020	0.0124
SEM(25)	4.1378	16.4464	29.3621
Source of Variation		**(P-value)**	
House Effect	< 0.0001	0.0414	< 0.0001
Diet Effect	0.1087	0.0007	0.0003
Diet × House	0.0002	0.0828	0.0058

[1] There were no significant differences in chick starting weights at 1 d of age (41 g).
[2] C: finely ground corn.
[3] CC: coarsely ground corn.
[4] T: finely ground triticale.
[5] WT: whole triticale.
[6] SEM(25): standard error of the mean with 25 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 4. Effect of housing design, grain type and particle size on periodic body gain of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	1 to 14 d	14 to 28 d	28 to 42 d	1 to 42 d
Litter House		**(g)**		
C[1]	364.5^{A}	1049.9	1403.7	2818.1^{A}
CC[2]	321.6^{C}	966.5	1356.9	2645.0^{B}
T[3]	351.4^{AB}	1051.2	1424.7	2827.3^{A}
WT[4]	328.9^{BC}	1000.3	13.56.1	2685.4^{B}
P-value	0.0053	0.1486	0.2930	0.0010
SEM(25)[5]	8.5297	29.5289	30.0472	33.9624
Broilermatic		**(g)**		
C	309.4^{b}	966.1	1290.8	2566.3^{b}
CC	320.3^{ab}	965.5	1320.9	2606.7^{b}
T	314.7^{b}	999.5	1398.9	2713.1^{a}
WT	326.5^{a}	958.9	1345.8	2631.2^{ab}
P-value	0.0383	0.1992	0.0092	0.0123
SEM(25)	4.0735	14.1655	20.9733	29.3487
Source of Variation		**(P-value)**		
House Effect	< 0.0001	0.0091	0.0148	< 0.0001
Diet Effect	0.1049	0.0559	0.0267	0.0003
Diet × House	0.0002	0.3628	0.2125	0.0059

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(25): standard error of the mean with 25 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 5. Effect of housing design, grain type and particle size on feed consumption of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	1 to 14 d	14 to 28 d	28 to 42 d	1 to 42 d
Litter House		**(g)**		
C[1]	449.3^{A}	1588.5	2786.5^{A}	4824.3^{A}
CC[2]	387.0^{C}	1524.8	2641.3^{C}	4553.0^{B}
T[3]	423.7^{AB}	1589.8	2900.6^{B}	4914.0^{A}
WT[4]	401.2^{BC}	1480.8	2595.8^{C}	4477.8^{B}
P-value	0.0096	0.0028	<0.0001	<0.0001
SEM(25)[5]	12.5427	21.3359	33.1032	49.2057
Broilermatic		**(g)**		
C	369.6	1533.7	2730.2	4633.5
CC	366.0	1489.1	2839.9	4695.0
T	380.1	1492.3	2936.4	4808.8
WT	376.1	1507.0	2974.6	4857.7
P-value	0.2191	0.8565	0.2896	0.5479
SEM(25)	5.0240	40.2102	95.0273	120.8313
Source of Variation		**(P-value)**		
House Effect	< 0.0001	0.0817	0.0079	0.3908
Diet Effect	0.0061	0.1528	0.0645	0.0694
Diet × House	0.0136	0.2930	0.0194	0.0140

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(25): standard error of the mean with 25 degrees of freedom.
[A,B,C] Means with different superscripts within a column differ significantly (P < 0.01).

Table 6. Effect of housing design, grain type and particle size on feed conversion ratio of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	1 to 14 d	14 to 28 d	28 to 42 d	1 to 42 d
Litter House		**(g/g)**		
C[1]	1.24	1.51	1.99	1.71
CC[2]	1.21	1.58	1.94	1.72
T[3]	1.21	1.51	2.04	1.74
WT[4]	1.22	1.50	1.94	1.67
P-value	0.9350	0.4320	0.4199	0.0596
SEM(25)[5]	0.0360	0.0388	0.0463	0.0171
Broilermatic		**(g/g)**		
C	1.19	1.58	2.11	1.80
CC	1.14	1.54	2.15	1.80
T	1.20	1.49	2.10	1.77
WT	1.15	1.57	2.21	1.85
P-value	0.0197	0.4256	0.6321	0.7890
SEM(25)	0.0158	0.0416	0.0641	0.0413
Housing Design		**(g/g)**		
Litter House	1.22[a]	1.53	1.98[B]	1.71[B]
Broilermatic	1.18[b]	1.55	2.14[A]	1.81[A]
SEM(50)[6]	0.0139	0.0201	0.0280	0.0158
Source of Variation		**(P-value)**		
House Effect	0.0332	0.4335	< 0.0001	< 0.0001
Diet Effect	0.4787	0.4887	0.9476	0.9969
Diet × House	0.5713	0.3610	0.2802	0.1811

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(25): standard error of the mean with 25 degrees of freedom.
[6] SEM(50): standard error of the mean with 50 degrees of freedom.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
[A,B] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 7. Effect of housing design, grain type and particle size on relative gizzard and proventriculus weights[1] of broilers fed triticale- or corn-based diets from 1 to 42 days of age

	Gizzard	Proventriculus
Dietary Treatments	**(g/kg)[6]**	
C[2]	19.38^{B}	5.83^{C}
CC[3]	23.32^{A}	6.05^{BC}
T[4]	19.73^{B}	6.37^{A}
WT[5]	23.51^{A}	6.25^{AB}
P-value	< 0.0001	0.0016
SEM(336)[6]	0.3523	0.1043
Housing Design	**(g/kg)**	
Litter Floor	22.18^{A}	6.21
Broilematic	20.79^{B}	6.03
P-value	< 0.0001	0.0878
SEM(336)	0.2491	0.0738
Source of Variation	**(P-value)**	
Age	< 0.0001	< 0.0001
Age × Diet	0.5884	0.1001
Age × House	0.0854	0.1482
Diet × House	0.1183	0.0636
Age × Diet × House	0.3625	0.7728
Corn × Triticale	0.4496	0.0004
Ground × Coarse	< 0.0001	0.6549

[1] Relative gizzard and proventriculus weights are means of organs weights of 8 birds per dietary treatment collected on days 7, 14, 21 and 28.
[2] C: finely ground corn.
[3] CC: coarsely ground corn.
[4] T: finely ground triticale.
[5] WT: whole triticale.
[6] SEM(336): standard error of the mean with 336 degrees of freedom.
[7] Relative organ weight: g of organ per kg of body weight.
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 8. Effect of housing design, grain type and particle size on gizzard and proventriculus pH of broilers fed triticale- or corn-based diets from 1 to 42 days of age

	14 d		21 d		28 d		35 d		42 d	
	Gizzard	**PV[6]**	**Gizzard**	**PV**	**Gizzard**	**PV**	**Gizzard**	**PV**	**Gizzard**	**PV**
Dietary Treatments					**(pH)**					
C[1]	3.21	4.12^{B}	4.01^{B}	4.46^{A}	4.72^{A}	4.56^{A}	4.78^{a}	5.25^{A}	4.51^{a}	4.98^{B}
CC[2]	3.20	4.51^{A}	3.92^{B}	4.65^{A}	4.23^{B}	4.48^{A}	4.40^{b}	5.23^{A}	4.16^{b}	5.35^{A}
T[3]	3.14	3.74^{C}	4.24^{A}	4.16^{B}	4.89^{A}	4.10^{B}	4.62^{ab}	4.66^{B}	4.41^{a}	4.91^{B}
WT[4]	3.29	4.35^{AB}	3.83^{B}	4.43^{A}	4.23^{B}	4.43^{A}	4.45^{b}	4.62^{B}	4.28^{ab}	4.93^{B}
P-value	0.4214	<0.0001	0.0008	0.0021	0.0002	0.0099	0.0486	0.0017	0.0349	0.0079
SEM(56)[5]	0.0648	0.0969	0.0699	0.0872	0.1208	0.0982	0.1113	0.1437	0.0867	0.1002
Housing Design					**(pH)**					
Litter Floor	3.14^{b}	4.20	3.78^{B}	4.19^{B}	4.18^{B}	4.77^{A}	4.44^{b}	4.88	4.33	5.03
Broilermatic	3.28^{a}	4.15	4.22^{A}	4.67^{A}	4.85^{A}	4.01^{B}	4.69^{a}	5.00	4.34	5.05
P-value	0.0358	0.5835	<0.0001	<0.0001	<0.0001	<0.0001	0.0287	0.4050	0.9058	0.8450
SEM(56)	0.0458	0.0685	0.0494	0.0616	0.0854	0.0694	0.0787	0.1016	0.0613	0.0709
Source of Variation					**(P-value)**					
Diet × House	0.1186	0.9263	0.0632	0.0588	0.0696	0.4684	0.0755	0.3545	0.8802	0.0684
Corn × Triticale	0.9311	0.0079	0.3359	0.0035	0.4670	0.0119	0.6232	0.0001	0.9115	0.0101
Ground × Coarse	0.2721	<0.0001	0.0006	0.0598	<0.0001	0.2210	0.0146	0.8354	0.0073	0.0565

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(56): standard error of the mean with 56 degrees of freedom.
[6] Proventriculus.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 9. Effect of housing design, grain type and particle size on relative total small intestine weight (g/kg)[1] and length (cm/kg)[2] of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	14 d		28 d		42 d	
	Wt[8]	Lg[9]	Wt	Lg	Wt	Lg
Litter House						
C[3]	50.62	265.51	36.52	90.97	23.46	52.12
CC[4]	42.63	271.82	35.65	103.62	21.53	52.88
T[5]	45.79	282.19	35.42	93.93	22.52	48.19
WT[6]	38.63	234.19	36.49	92.82	22.53	54.43
P-value	0.0928	0.2396	0.9307	0.2621	0.6157	0.3629
SEM(28)[7]	3.2979	16.9518	1.4797	4.7716	1.0109	2.5264
Broilermatic						
C	36.92	234.20	38.98	92.53	26.54	61.31
CC	42.74	263.86	38.47	97.53	23.86	61.05
T	41.46	262.56	37.79	97.93	24.26	54.48
WT	39.41	239.63	37.51	92.17	23.36	53.05
P-value	0.1477	0.3321	0.9359	0.5682	0.1029	0.0695
SEM(28)	1.8311	14.0873	1.7826	3.7604	0.9340	2.6642
Source of Variation			**(P-value)**			
House Effect	0.0270	0.2304	0.0669	0.9238	0.0054	0.0037
Diet Effect	0.2561	0.0960	0.9170	0.1658	0.0911	0.1081
Diet × House	0.0327	0.6767	0.9506	0.6828	06991	0.1773

[1] Relative small intestine weight: g of total small intestine per kg of body weight.
[2] Relative small intestine length: cm of total small intestine per kg of body weight.
[3] C: finely ground corn.
[4] CC: coarsely ground corn.
[5] T: finely ground triticale.
[6] WT: whole triticale.

Table 9. Continued

[7] SEM(28): standard error of the mean with 28 degrees of freedom.

[8] Relative weight (g/kg).

[9] Relative length (cm/kg).

[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

[A,B] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 10. Effect of housing design, grain type and particle size on relative small intestinal weight of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	14 d			28 d			42 d		
	D[6]	J[7]	I[8]	D	J	I	D	J	I
Litter House					(g/kg)[9]				
C[1]	12.32	22.54[a]	15.20	7.82	16.13	12.57	5.38	9.98	8.11
CC[2]	11.55	17.32[b]	13.21	8.37	15.59	11.69	5.39	9.29	6.84
T[3]	12.05	19.24[ab]	13.91	7.70	15.64	12.09	5.48	9.89	7.15
WT[4]	9.56	15.97[b]	12.58	7.40	16.16	12.94	5.36	9.62	7.55
P-value	0.125	0.019	0.439	0.444	0.912	0.749	0.990	0.734	0.300
SEM(28)[5]	0.863	1.444	1.166	0.427	0.729	0.858	0.290	0.465	0.484
Broilermatic					(g/kg)				
C	9.43	15.15	11.95	8.83	16.35	13.81	6.53	11.16	8.85
CC	11.24	17.88	13.05	7.74	16.88	13.85	5.24	9.95	8.67
T	10.45	18.88	11.57	7.65	17.06	13.08	5.50	10.24	8.55
WT	9.87	15.93	13.28	8.10	17.02	12.39	4.94	9.77	8.64
P-value	0.197	0.065	0.175	0.270	0.944	0.510	0.092	0.135	0.981
SEM(28)	0.606	1.048	0.625	0.457	0.929	0.779	0.449	0.434	0.510
Source of Variation					(P-value)				
House Effect	0.058	0.048	0.061	0.412	0.114	0.103	0.598	0.071	0.001
Diet Effect	0.113	0.064	0.829	0.437	0.970	0.885	0.180	0.152	0.474
Diet × House	0.156	0.008	0.133	0.249	0.888	0.426	0.182	0.678	0.731

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(28): standard error of the mean with 28 degrees of freedom.
[6] Duodenum.

Table 10. Continued

[7] Jejunum.

[8] Ileum.

[9] Relative tissue weight: g of tissue per kg of body weight.

[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 11. Effect of housing design, grain type and particle size on relative small intestinal length of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	14 d			28 d			42 d		
	D[6]	J[7]	I[8]	D	J	I	D	J	I
Litter House					(cm/kg)[9]				
C[1]	45.6	95.1	126.0	15.8	35.8	39.3	10.3	20.9	20.9
CC[2]	44.8	107.5	119.6	18.1	41.4	44.1	10.9	20.9	21.1
T[3]	48.3	109.4	124.4	16.1	37.6	40.2	10.9	18.6	18.7
WT[4]	40.1	90.9	103.2	16.7	39.1	37.1	11.4	22.1	21.0
P-value	0.223	0.075	0.358	0.152	0.391	0.197	0.601	0.300	0.329
SEM(28)[5]	2.718	5.723	9.880	0.737	2.262	2.260	0.534	1.280	1.057
Broilermatic					(cm/kg)				
C	42.3	84.9	107.1	17.8	36.0	38.7	12.3	24.3	24.7
CC	45.6	101.0	117.4	17.5	39.0	41.0	11.6	24.5	25.0
T	44.4	100.4	118.1	18.4	39.4	40.1	11.4	21.5	21.5
WT	42.7	88.8	108.1	17.5	37.9	36.8	11.1	20.9	21.1
P-value	0.769	0.214	0.602	0.789	0.470	0.456	0.460	0.113	0.057
SEM(28)	2.452	6.452	7.383	0.721	1.629	1.948	0.533	1.247	1.230
Source of Variation					(P-value)				
House Effect	0.606	0.112	0.369	0.037	0.784	0.502	0.064	0.017	0.002
Diet Effect	0.279	0.014	0.307	0.563	0.199	0.072	0.995	0.146	0.036
Diet × House	0.534	0.914	0.582	0.200	0.735	0.886	0.226	0.207	0.311

[1] C: finely ground corn.
[2] CC: coarsely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.
[5] SEM(28): standard error of the mean with 28 degrees of freedom.
[6] Duodenum.

Table 11. Continued

[7] Jejunum.

[8] Ileum.

[9] Relative tissue length: cm of tissue per kilogram of body weight.

[A,B] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 12. Effect of housing design, grain type and particle size on relative ceca weight (g/kg)[1] and length (cm/kg)[2] of broilers fed triticale- or corn-based diets from 1 to 42 days of age

TREATMENT	14 d		28 d		42 d	
	Wt[8]	Lg[9]	Wt	Lg	Wt	Lg
Litter House						
C[3]	7.84	21.53	4.19	7.84	3.76	5.14
CC[4]	8.23	20.37	4.20	9.12	3.60	5.98
T[5]	8.62	19.31	4.69	8.07	3.54	6.10
WT[6]	8.67	18.56	4.36	8.52	4.05	6.41
P-value	0.9109	0.4235	0.5223	0.4337	0.4083	0.0447
SEM(28)[7]	0.9246	1.3145	0.2671	0.5776	0.2286	0.3099
Broilermatic						
C	9.06	16.15[b]	5.05	8.78	4.11	6.07
CC	8.69	19.09[a]	5.17	8.66	4.75	6.04
T	7.59	20.05[a]	5.27	9.45	4.06	5.68
WT	8.28	17.94[ab]	5.95	9.26	4.75	6.73
P-value	0.6284	0.0235	0.3846	0.6556	0.5536	0.3681
SEM(28)	0.8255	0.8741	0.3924	0.5083	0.4526	0.4155
Source of Variation			**(P-value)**			
House Effect	0.9197	0.0425	<0.0001	0.0966	0.0096	0.3905
Diet Effect	0.9694	0.4918	0.3479	0.6798	0.3618	0.0762
Diet × House	0.5950	0.0491	0.4977	0.3858	0.7177	0.3270

[1] Relative ceca weight: g of ceca (arithmetic mean of both cecum) per kg of body weight.
[2] Relative ceca length: cm of ceca (arithmetic mean of both cecum) per kg of body weight.
[3] C: finely ground corn.
[4] CC: coarsely ground corn.
[5] T: finely ground triticale.
[6] WT: whole triticale.

Table 12. Continued

[7] SEM(28): standard error of the mean with 28 degrees of freedom.

[8] Relative weight (g/kg).

[9] Relative length (cm/kg).

[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 13. Effect of housing design, grain type and particle size on histological measurements[1] of jejunum of 3 day old broilers fed triticale- or corn-based diets

	Villus				Crypt Depth	Villus:Crypt Ratio	Mucosal Depth	Muscularis Depth
	Height	Apical Width	Basal Width	Area				
Dietary Treatments		(µm)		(mm^2)	(µm)	(µm/µm)	(µm)	
C[2]	380.31	55.68	98.54	29.72	73.50	5.21	453.82	75.16
CC[3]	402.45	53.03	90.91	29.17	75.75	5.33	478.20	71.64
T[4]	413.99	61.02	102.82	33.71	76.54	5.45	490.53	73.76
WT[5]	365.18	56.87	94.79	27.63	72.32	5.12	437.50	68.22
P-value	0.1068	0.1239	0.4020	0.1702	0.5466	0.7471	0.0944	0.4311
SEM(53)[6]	14.2705	2.1761	4.8051	1.7981	2.2099	0.2109	15.1819	3.0925
Housing Design		(µm)		(mm^2)	(µm)	(µm/µm)	(µm)	
Litter House	412.33^{A}	58.38	99.56	32.77^{A}	73.94	5.63^{A}	486.26^{A}	72.27
Broilermatic	368.64^{B}	54.92	93.97	27.35^{B}	75.12	4.92^{B}	443.76^{B}	72.13
P-value	0.0047	0.1315	0.2666	0.0053	0.6067	0.0018	0.0093	0.9655
SEM(53)	10.8211	1.6501	3.6436	1.3635	1.6757	0.1599	11.5123	2.3450
Source of Variation					(P-value)			
Diet × House	0.0595	0.2314	0.9546	0.3100	0.7270	0.3671	0.0621	0.3930
Corn × Triticale	0.9037	0.0468	0.4165	0.5146	0.9328	0.9458	0.8997	0.4574
Ground × Coarse	0.3715	0.1377	0.1221	0.0808	0.6676	0.6273	0.3669	0.1629

[1] Histological measurements:
Villus height = distance between the villus tip to the villus base, not including the intestinal crypt;
Villus apical width = measurement of the width of the villus tip;
Villus basal width = measurement of the width of the villus at the crypt-villus junction;
Villus surface area = [(villus apical width + villus basal width)/2] × villus height;
Villus:crypt ratio = villus height divided by crypt depth;
Crypt depth = distance between the villus base to the muscularis, not including intestinal muscularis;

Table 13. Continued

Mucosal depth = Villus height + crypt depth;

Muscularis depth = distance between the crypt to serosa layer, not including intestinal serosa.

[2] C: finely ground corn.

[3] CC: coarsely ground corn.

[4] T: finely ground triticale.

[5] WT: whole triticale.

[6] SEM(53): standard error of the mean with 53 degrees of freedom.

[A,B] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 14. Effect of housing design, grain type and particle size on carcass yields[1] of 42 days old broilers fed triticale- or corn-based diets from 1 to 42 days of age

	Live Wt[7]	Heart	Liver	Gizzard	Int[8]	Abd Fat[9]	Evisc Carc[10]	Iced Carc[11]	Drums	Thighs	Wings	Breast[12]	Meat Yield[13]
Dietary Treatments	**(kg)**						**(g/kg)[14]**						
C[2]	2.68	5.55	18.21	9.34^{B}	46.63	16.81^{A}	760.00^{AB}	784.92	99.69	129.05	84.49	204.68	517.91
CC[3]	2.64	5.46	17.39	13.16^{A}	46.55	17.07^{A}	752.05^{C}	789.53	99.65	129.32	83.96	199.35	512.27
T[4]	2.76	5.26	17.43	9.01^{B}	46.89	11.61^{B}	763.84^{A}	805.71	104.15	127.48	84.62	215.39	531.63
WT[5]	2.56	5.40	17.43	13.53^{A}	45.47	14.37^{AB}	752.48^{BC}	792.31	101.50	128.69	85.50	202.37	518.06
P-value	0.1252	0.7225	0.5684	<0.0001	0.7771	0.0008	0.0091	0.1412	0.3981	0.9370	0.8569	0.0046	0.0333
SEM(120)[6]	0.0580	0.1829	0.4854	0.3327	1.0354	1.0466	2.8881	6.5587	2.1268	2.1900	1.2645	3.2672	4.7486
Housing Design	**(kg)**						**(g/kg)**						
Litter Floor	2.68	5.38	18.28^{A}	11.81^{A}	46.11	16.11^{a}	759.69	795.98	100.30	125.64^{B}	84.08	208.61	518.63
Broilermatic	2.64	5.46	16.94^{B}	10.71^{B}	46.66	13.82^{b}	754.49	790.26	102.19	131.63^{A}	85.21	202.28	521.30
P-value	0.4701	0.6697	0.0068	0.0012	0.5949	0.0304	0.0743	0.3855	0.3767	0.0072	0.3728	0.0548	0.5749
SEM(120)	0.0410	0.1293	0.3433	0.2352	0.7321	0.7400	2.0422	4.6377	1.5039	1.5486	0.8941	2.3102	3.3578
Source of Variation							**(P-value)**						
Diet × House	0.3062	0.2253	0.4514	0.9331	0.4488	0.5078	0.7640	0.3461	0.7363	0.2523	0.3363	0.0185	0.0500
Corn × Triticale	0.9976	0.3365	0.4427	0.9535	0.6889	0.0002	0.4619	0.0749	0.1401	0.6162	0.5113	0.0378	0.0422
Ground × Coarse	0.0436	0.8890	0.3977	<0.0001	0.4706	0.1506	0.0011	0.5047	0.5279	0.7343	0.8917	0.0058	0.0455

[1] Yields were obtained from a total of 128 broilers processed (16 birds/treatment/house).
[2] C: finely ground corn.
[3] CC: coarsely ground corn.
[4] T: finely ground triticale.
[5] WT: whole triticale.
[6] SEM(120): standard error of the mean with 120 degrees of freedom.
[7] Broiler live weight.

Table 14. Continued

[8] Intestines: includes small and large intestines.
[9] Abdominal fat.
[10-11] Eviscerated and Iced carcass, respectively.
[12] Breast: sum of relative weights of pectoralis major and minor.
[13] Meat yield: sum of relative weights of drumsticks, thighs, wings and breast.
[14] Relative weights: g of part or organ per kg of body weight.
[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.01$).

Table 15. Effect of housing design, grain type and particle size on relative breast[1] weight and meat yield[2] of 42 days old broilers fed triticale- or corn-based diets

TREATMENT	Breast	Meat Yield
Litter House	**(g/kg)**	
C[3]	214.10	524.69
CC[4]	206.44	515.62
T[5]	214.96	526.30
WT[6]	198.94	507.92
P-value	0.0938	0.2319
SEM(60)[7]	5.0204	7.0541
Source of Variation	**(P-value)**	
Corn × Triticale	0.5108	0.6676
Ground × Coarse	0.0216	0.0563
Broilermatic	**(g/kg)**	
C	195.25^{BC}	511.12^{BC}
CC	192.26^{C}	508.93^{C}
T	215.80^{A}	536.95^{A}
WT	205.80^{AB}	528.21^{AB}
P-value	0.0006	0.0063
SEM(60)	4.1825	6.3590
Source of Variation	**(P-value)**	
Corn × Triticale	0.0001	0.0008
Ground × Coarse	0.1256	0.3933

[1] Breast: sum of relative weights of pectoralis major and minor. Yields were obtained from a total of 128 broilers processed (16 birds/treatment/house).
[2] Meat yield: sum of relative weights of drumsticks, thighs, wings and breast.
[3] C: finely ground corn.
[4] CC: coarsely ground corn.
[5] T: finely ground triticale.
[6] WT: whole triticale.
[7] SEM(60): standard error of the mean with 60 degrees of freedom.
[A,B,C] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 16. Pearson correlation coefficients (r) of gizzard and proventriculus relative weight and pH, relative ceca weight and length, and cecal *Salmonella* population of broilers reared in a litter house or Broilermatic non-litter cage system and fed triticale- or corn-based diets

Correlation Values	Correlation Coefficient (r)	P-value
RCWT[1] vs. *Salmonella*	+0.30	< 0.0001
RCL[2] vs. *Salmonella*	+0.12	0.0251
GpH[3] vs. PVpH[4]	+0.37	< 0.0001
RGWT[5] vs. RPVWT[6]	+0.87	< 0.0001
RGWT vs. GpH	-0.62	< 0.0001
RPVWT vs. PVpH	-0.29	< 0.0001

[1] Relative ceca weight: g of ceca per kg of bird body weight.
[2] Relative ceca length: cm of ceca per kg of bird body weight.
[3] Gizzard pH.
[4] Proventriculus pH.
[5] Relative gizzard weight: g of empty gizzard per kg of bird body weight.
[6] Relative proventriculus weight: g of empty proventriculus per kg of bird body weight.

Table 17. Fecal *Salmonella* populations of broilers fed triticale- or corn-based diets and raised in a conventional litter floored house or in the Broilermatic non-litter cage system

	1 d[1]	14 d	28 d
Dietary Treatments		**log MPN/g**	
C[2]	1.98	6.88	6.13
CC[3]	2.35	6.97	5.89
T[4]	2.43	6.90	5.91
WT[5]	2.75	6.72	5.78
P-value	0.1543	0.7871	0.0973
SEM (50)[6]	0.2474	0.1858	0.1507
Housing Design		**log MPN/g**	
Litter Floor	2.24	6.56^{B}	5.32^{B}
Broilermatic	2.52	7.18^{A}	6.45^{A}
P-value	0.2669	0.0010	0.0023
SEM (50)	0.1749	0.1270	0.1300
Source of Variation		**(P-value)**	
House × Diet	0.4966	0.6932	0.9160

[1] Meconium samples: pooled meconium of 40 chicks collected prior to *Salmonella* challenge.
[2] C: finely ground corn.
[3] CC: coarsely ground corn.
[4] T: finely ground triticale.
[5] WT: whole triticale.
[6] SEM (50): standard error of the mean with 50 degrees of freedom.
[A,B] Means with different superscripts within a column differ significantly ($P < 0.01$).

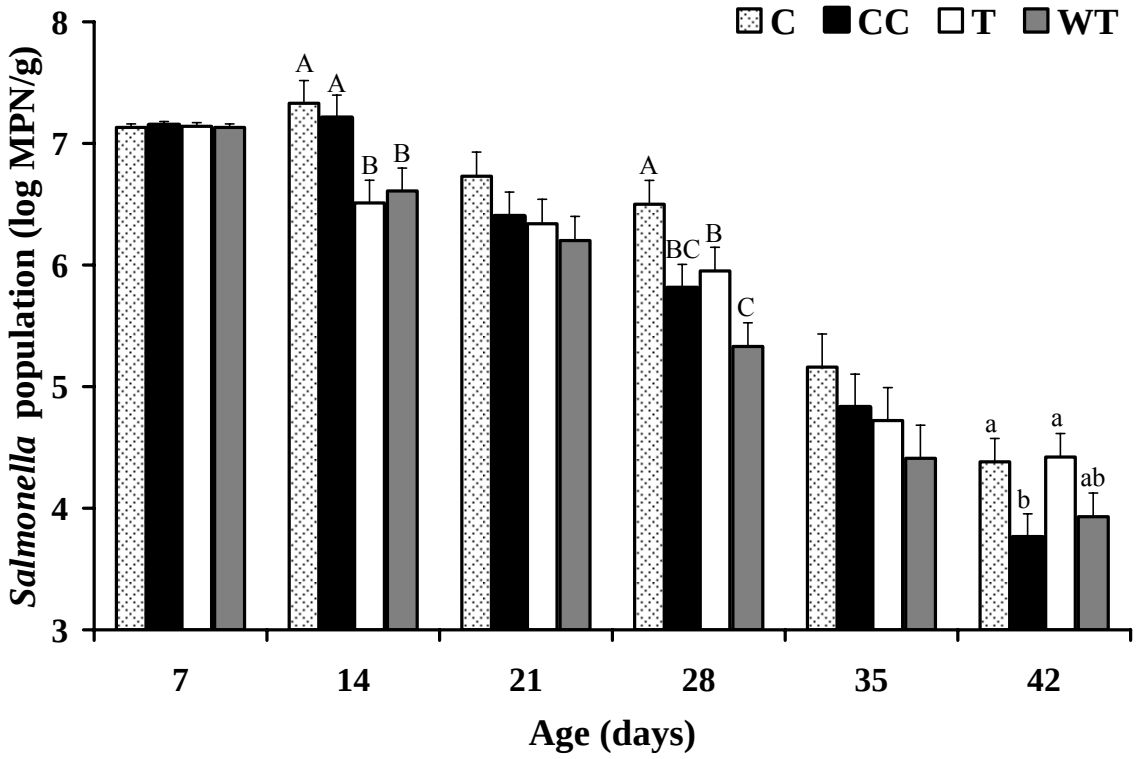

Figure 1. Effect of dietary treatments on *Salmonella* cecal populations of broilers fed finely ground corn (C), coarsely ground corn (CC), finely ground triticale (T) or whole triticale as crumble form (1-14 d) or pellet form (15-42 d). Means with different superscript letters within an age group differ significantly (^{a,b} $P < 0.05$, ^{A,B,C} $P < 0.01$).

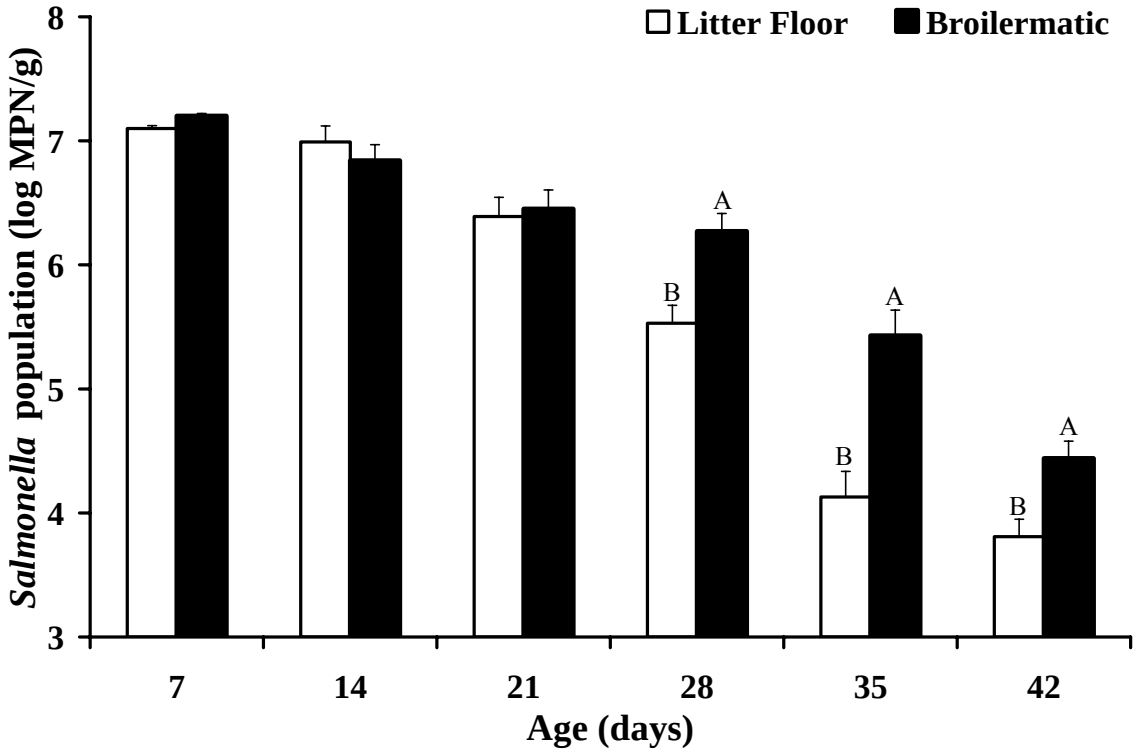

Figure 2. Effect of housing design on *Salmonella* cecal populations of broilers raised in a conventional litter floored house or in the Broilermatic non-litter cage system. Means with different superscript letters within an age group differ significantly (^{A,B} $P < 0.01$).

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CHAPTER 6

DETERMINATION OF ILEUM MICROBIAL DIVERSITY BY DENATURING GRADIENT GEL ELECTROPHORESIS ANALYSIS OF 16S RIBOSOMAL DNA AMPLICONS OF BROILERS FED TRITICALE- OR CORN-BASED DIETS AND COLONIZED BY *SALMONELLA*[*]

[*] Use of trade names in this publication does not imply endorsement by the North Carolina Agriculture Research Service or the North Carolina Cooperative Extension Service of the products mentioned, nor criticism of similar products not mentioned.

6.1 SUMMARY

Diversity of the bacterial communities in the ileum of broilers was characterized using denaturing gradient gel electrophoresis (DGGE). DGGE separation of polymerase chain reaction (PCR) amplicons of the V2-V3 variable regions of the 16S rDNA is a common method to profile community diversity and has been used to assess the effects of diet and antibiotics on the ileal bacterial community of chickens. Broilers raised either on litter floor or in cage batteries were fed either a finely ground corn- (control), a finely ground triticale- or a whole triticale-based diet from 0-42 d. Microbial DNA was extracted from the ileum content of 42 d broilers and the 16S rDNA gene was amplified by PCR and the amplicons separated by DGGE. Diversity indexes including richness, evenness, diversity, and pairwise similarities coefficient were calculated. Diversity indexes were related to the dietary treatments, housing designs and with changes in *Salmonella* colonization of broiler ceca as characterized by the most probable number method (MPN). Higher microbial diversity indexes were observed among birds fed whole triticale-based diets and reared on litter floor. In contrast, finely ground grain treatments had lower diversity and higher *Salmonella* prevalence than the whole triticale treatment. The combination of high dietary fiber content and increased coarseness of the diet by feeding whole triticale presumably stimulated microbial community diversity and discouraged *Salmonella* colonization through a competitive exclusion type mechanism.

Key words: *Salmonella*, broilers, microbial diversity, triticale, whole grain

6.2 DESCRIPTION OF PROBLEM

The relationship between the intestinal microflora and the host is not merely commensal, but rather symbiotic or mutualistic, considering that this association is generally beneficial for both bacteria and host. The intestinal flora generally impacts the host's health by influencing digestion and nutrient absorption, intestinal morphology, and defense of the host against infection (Abrams et al., 1963; Mead, 2000). This protective effect is accomplished by inhibitory effects exerted by the resident microflora including competition for nutrients (Hungate, 1966), production of antimicrobial substances such as bacteriocins (Jack et al., 1995), and physical binding to the surface of the intestinal epithelium, preventing opportunistic pathogens from obtaining an attachment site along the intestinal mucosa (Collins and Gibson, 1999). The composition and diversity of the microbial community of the avian intestinal tract can be influenced by many factors including bird age (Lu et al., 2003; Amit-Romach et al., 2004), intestinal infections (Kimura et al., 1976), and diet (Netherwood et al., 1999; Lu et al., 2003).

Promoting the development of the beneficial bacteria within the avian intestinal tract could help to reduce foodborne pathogen colonization, which would reduce human exposure to these pathogenic organisms and related illness and deaths (Callaway et al., 2003). It is well recognized that some antimicrobials can improve growth and health of broilers and turkeys. Several of these antibiotics provide significant protective effect against *Salmonella* colonization and shedding of poultry, but their level of efficacy can vary depending on dosage, combination of products (De Oliveira et al., 2000), and pre-exposure to *Salmonella* (Bailey et al., 1998). Although antimicrobial drugs can improve

growth performance and some may be effective against *Salmonella* colonization of the poultry gastrointestinal tract (GIT) (Bolder et al., 1999), the concern over resistance to antibiotics that are commonly used in human medicine has lead to the restriction of these drugs for use as animal feed additives.

Several alternative products intended to control *Salmonella* in poultry have been developed in response to the anticipated reduction in using antibiotics as growth promoters. Other nutritional strategies that have been used in the poultry industry to promote intestinal health and to increase the resistance to pathogen colonization include the use of probiotics, prebiotics, whole grain and enzyme supplementation. Netherwood et al. (2003) monitored the response of the avian intestinal bacterial microflora to probiotic administration and detected a shift in the composition of the microflora after probiotic use. Prebiotics, which are carbohydrates or other organic compounds that are not digestible by the host animal but digestible by specific microbial populations of the intestinal tract (Callaway et al., 2003), have also been used to promote intestinal health of poultry (Lowry et al., 2005).

Cereals such as wheat and triticale are rich in non-starch polysaccharides (NSP) that comprise a major part of dietary crude fiber. NSP are a heterogeneous group of polysaccharides having varying degrees of water solubility, size, and structure and are not digested by the avian digestive tract (Lineback and Rasper, 1988). NSP have been used for many years in poultry diets as dietary fiber and more recently they have been evaluated as potential prebiotics (Santos, 2006). For example, dietary inclusion of β-glucan will stimulate microbial fermentation and to protect broiler chickens against

Salmonella colonization by up-regulating heterophil phagocytosis, bactericidal killing and oxidative burst (Lowry et al., 2005). Cereals can also be added as whole grains to poultry diets to improve growth performance of broilers (Bennett et al., 2002a; Svihus et al., 2004), turkeys (Bennett et al., 2002b; Santos, 2006), and layers (Lázaro et al., 2003). Whole grain feeding has also been shown to shift intestinal microflora towards a more healthy state by reducing *Salmonella* prevalence in the gizzard and ileum of broilers (Bjerrum et al., 2005). Similarly, the *Salmonella* death rate in gizzard contents is greater in broilers fed coarse mash feed than those fed fine mash or pelleted diets (Huang et al., 2006).

High fiber content diets are commonly supplemented with fiber-degrading enzymes. Supplemental enzymes in the feed are frequently used to alleviate the adverse effects of antinutritional factors (Santos, 2006), render certain nutrients more available for absorption, enhance the energy value of feed ingredients (Lyons and Walsh, 1993), and modulate intestinal microflora to a healthier state (Engberg et al., 2004; Högberg and Lindberg, 2004). The use of non-starch polysaccharide degrading enzymes is now very common in cereal-based poultry diets. Most fungal and bacterial enzyme preparations effectively degrade the viscous polysaccharides (e.g. β-glucan, arabinoxylan) found in barley, oats, rye and wheat (Jensen et al., 1957, Odetallah et al., 2002; Silva and Smithard, 2002). Dietary supplementation of endoxylanase, a NSP-degrading enzyme, inhibits the proliferation of fermentative microorganisms in the small intestine by increasing the digesta passage rate and nutrient digestion (Choct et al., 1999). Thus, nutrient utilization is improved by reducing the competition between the host and its

enteric microflora. Although microbial fermentation in the small intestine decreases after dietary endoxylanase supplementation, microbial fermentation increases in the large intestine and ceca as a result of increased substrate in the lower intestine (Steenfeldt et al., 1998).

Prolonging infections through the recycling of pathogens from direct contact with litter is another concern of the poultry industry. Poultry litter has been identified as a potential source of contamination of foodborne pathogens for poultry, especially *Salmonella* (Bryan et al., 1979). The land application of contaminated litter may also pose a potential health risk to humans and other animals *via* contamination of ground and surface waters or crops (Nicholson et al., 2005). An alternative strategy for pathogen control and growth promotion is the rearing of broilers and turkeys on non-litter systems. The Farmer Automatic Broilermatic Cage System is an example of a non-litter system. The system is a cage facility that allows broilers to be raised on a surface nearly free of feces, yet avoiding many of the problems associated with standard cage systems such as breast blisters, folliculitis, and wing and leg breakage/downgrade problems (Havenstein et al., 1998).

The study reported herein was designed to evaluate the effects of grain, particle size and type housing system on the intestinal microbial diversity in broilers. It was hypothesized that broilers fed a triticale-based diet containing high NSP content, would have greater intestinal microbial community diversity and less *Salmonella* colonization, than broilers fed a corn-based diet. It was further hypothesized that the beneficial effect of feeding triticale would be maximized by the use of whole grains. The experiment was

also designed to test the effect of housing systems on microbial community diversity and *Salmonella* colonization of the small intestine of broilers. The cage system allows broilers to be raised on a surface nearly free of feces which was assumed to reduce recontamination of birds with *Salmonella* present in their fecal matter. In contrast, it was anticipated that birds reared on litter would be more heavily colonized by *Salmonella* which would discourage the growth of some alternative and beneficial bacterial species thus compromising the intestinal microbial diversity. To test these hypotheses, changes in ileal bacterial populations of broilers fed triticale- or corn-based diets were investigated by the separation of 16S ribosomal DNA (rDNA) polymerase chain reaction (PCR) amplicons by denaturing gradient gel electrophoresis (DGGE). In addition, the changes in the intestinal microbial community diversity characterized by PCR-DGGE were related to changes in *Salmonella* colonization of turkeys. *Salmonella* fecal and cecal populations were determined by the most probable number method (MPN).

6.3 MATERIALS AND METHODS

6.3.1 Bird Husbandry

One thousand nine hundred and twenty one-day-old Ross 508 (Aviagen, Huntsville, AL) broiler chickens were weighed, neck-tagged and orally gavaged with 1 ml (8×10^5 colony forming units – cfu) of a cocktail of 4 serovars of *Salmonella enterica* subspecies *enterica* before being randomly assigned to 2 experimental housing designs, a conventional litter floored house or the Broilermatic System (Farmer Automatic of America Inc., Register, GA), a non-litter cage-based design (Havenstein et al., 1998). In

each house, broilers were assigned to1 of 3 dietary treatments consisting of finely ground corn for treatment 1 (C, control), and finely ground or whole triticale for treatments 2 (T) and 3 (WT), respectively. Birds were kept on a 24-day light schedule in both houses. Feed and water were provided *ad libitum*. Broilers were inspected daily and birds with visual health problems were removed and euthanized by cervical dislocation.

6.3.2 Experimental Design and Diets

The experimental design consisted of three dietary treatments (C, T, WT), each with eight replicate pens or cages of 40 broiler chickens per housing design. Over the entire experimental period (1-42 d), all broilers were fed either a corn- or a triticale-soybean meal (SBM) based diet. From 1-14 d of age (starter diet), feed was offered in crumble form and from 15-42 d (grower and finisher diets) in pellet form (Table 1). The experimental diets were formulated using least-cost linear programming to meet or exceed the NRC (1994) nutrient requirements. The corn was supplied and ground by Southern States Feed Mill (Siler City, NC), resulting in a final average particle size of 560 μm. The triticale (Tritical-498, Lot No TC-1101-B, Virginia, USA) was supplied by Resource Seeds (Gilroy, CA) and ground at the North Carolina State University feed mill (Raleigh, NC) in a hammermill (Bliss Industries Inc., Ponca City, OK) equipped with a 3-mm screen to produce a final average particle size of 560 μm. The third experimental diet was prepared using the whole triticale grain. Average particle size of the pelleted diets was 3.39, 4.25 and 2.99 mm for C, T, and WT, respectively. As recommended by the supplier, triticale-based diets were supplemented with Avizyme 1502 (Danisco Animal

Nutrition, Marlborough, UK) which provided 600 endo-1,4-beta-xylanase units (EXU, EC 3.2.1.8) per kg of feed. EXU is defined as the enzyme activity required to liberate 1 μmol of reducing sugar (measured as glucose equivalents) per minute from a 1% xylan solution at pH 3.5 and 40°C. The enzyme preparation also contained standardized activities of at least 8000 units of subtilisin (EC 3.2.1.8) and 800 units of alpha-amylase (EC 3.4.21.62) per gram of product. The feed did not contain any antimicrobials or coccidiostats.

6.3.3 Inoculum Preparation and Salmonella Enumeration

A cocktail of *Salmonella enterica* subspecies *enterica* serotypes Typhimurium (ATCC 700408), Newport (ATCC 6962), Heidelberg (ATCC 8326), and Kentucky (field isolate) was used as the inoculum. The serovar Kentucky had been previously isolated from turkey feces and was serotyped by the NVSL (National Veterinary Service Laboratories, Animal and Plant Health Inspection Services, USDA, Ames, IA). For the purpose of this document, serovars of *Salmonella enterica* subsp. *enterica* will be referred to as *Salmonella* accompanied by the serovar name (i.e. *Salmonella* Typhimurium or *S.* Typhimurium). For preparation of inoculum, the four serovars were grown separately overnight at 37°C in brain-heart infusion (BHI) broth (Oxoid Ltd., Ogdensburg, NY). The cultures were then mixed together and serially diluted in buffered peptone water (BPW, Oxoid Ltd., Ogdensburg, NY) to a final concentration of 8×10^5 cfu/ml. The cell count was determined by direct plating on BHI agar plates. Plates were

incubated overnight at 37°C. Negative controls were used for all plating procedures to ensure that the media had been properly sterilized.

At 42 days of age, 8 birds per dietary treatment per house were euthanized by cervical dislocation and the ceca were aseptically removed, weighed, and stored on ice for *ca*. 1 h before being quantitatively cultivated for *Salmonella* isolation. Cultivation of *Salmonella* was performed immediately after sampling using the most probable number (MPN) procedure as previously described (Santos et al., 2005). Populations of *Salmonella* for each sample were determined using Thomas' approximation (Swanson et al., 2001).

6.3.4 Bacterial DNA Isolation and PCR-DGGE Analysis

Fifteen centimeters of the distal part of the ileum (above the ileo-cecal-colonic junction) were aseptically removed from the same birds used for *Salmonella* enumeration and frozen at -20°C until further analysis.

To isolate bacterial DNA, ileum samples were first defrosted and their contents removed and pooled to reduce individual variation. Each pool was composed of the ileal contents of 4 birds per treatment per house. Bacteria were subsequently collected from the pooled sample using differential centrifugation. Community DNA from the bacterial suspensions was extracted using a ballistic lysis method (Lu et al., 2003). Briefly, lysed cells were treated with sodium dodecyl sulfate (final concentration, 0.5%) and proteinase K (final concentration, 0.1 mg/ml) and incubated at 37°C for 30 min. The sample was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1)

and once with chloroform-isoamyl alcohol (24:1). DNA was concentrated with a 0.6 volume of isopropanol and resuspended in 50 μl sterile water. DNA concentrations were measured with a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). For PCR amplification, each DNA sample was amplified using primers HDA1-GC and HDA2 as described by Walter et al. (2000). Diversity of the communities was characterized by separation of PCR amplicons using DGGE analysis as described Knarreborg et al. (2002) with some modifications. DGGE was performed using a 16 cm by 16 cm by 1mm-thick 6% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) containing a 15 to 55% gradient of urea and formamide. Amplicons were separated by electrophoresis at 200 V for 3 h and gels were stained using Sybr Green (Invitrogen, Carlsbad, CA). In order to compare gels run in different trials, samples were repeated on subsequent gels allowing alignment of the gels using band patterns from the repeated samples.

6.3.5 Examination of DGGE Gels

Examination of DGGE gels was based on methods previously described by McCracken et al. (2001), Konstantinov et al. (2003), and Høj et al. (2005). Gels were compared using the BioNumerics software (BioNumerics version 3.5, Applied Maths BVBA, Austin, TX) as follows. The number of bands per lane were assessed using a band searching algorithm within the program. A manual check was done and the DGGE fragments constituting less than 1% of the total area of all bands were omitted. Bands constituting 1% or more of the total area of all bands were considered as dominant

DGGE bands and included in the analysis. Subsequently, band migration distance and intensity of the bands within each lane of the gel were measured (Simpson et al., 2000). This information was then used to calculate several measures of microbial ecology including number of bands (microbial community richness, S), Shannon's Equitability (microbial community evenness, E_H) and Shannon's index (microbial community diversity, H') (Shannon and Weaver, 1949; Sneath and Sokal, 1973; Magurran, 1988). These indices measure ecological diversity using various parameters including species richness (the number of different species in the ecosystem) and evenness (the distribution of individuals within each species in the ecosystem) (Magurran, 1988). In the description of the indices that follows, "species" refers to individual bands on the DGGE gels. However, because the bands on the DGGE gels correspond to the percentage of G + C content within the melting domains for the V2 and V3 PCR amplicons, bacterial species with similar G + C content in the amplified V2 and V3 region may form assemblages and appear as a single band (Muyzer and Smalla, 1998).

The Shannon index was calculated using the following function: $H'=-\Sigma P_i \log Pi$, where P_i is the proportion of individuals in the population belonging to the i^{th} species, thus P_i corresponds to the proportional abundance of band *i.* Therefore, H' values were calculated based on the intensity of each band, as measured by the peak height of the band in the densitometric curves. The importance probability, P_i, was calculated as: $P_i=n_i/T$, where n_i is the peak height and T is the sum of all peak heights in the densitometric curve for a specific lane. Community evenness was calculated using the Shannon's equitability index (E_H index) based on the formula: $E_H = H'/\ln S$, where H' is

the Shannon's index and lnS is the natural log of the total number of species in the community (total number of bands in the DGGE gel, richness) (Foucher et al., 2004).

Band surface area corresponds to measurements of the optical density of each band. The optical densities were measured based on the plotted band intensity and migration distance. Each band formed a peak relative to its intensity and migration distance and the area underneath the peaks was measured using the BioNumerics software. Number of bands (richness, S) corresponds to the number of individual bands in a single lane. Sorenson's similarity index or coefficient of similarity index (C_S) was used to compare average percentage similarities of DGGE banding patterns within each treatment group (intragroup comparison) and between treatment groups (intergroup comparison). Calculations for the Sorenson's similarity index are based on the formula: $C_S = [2j/(a+b)]\times 100$, where a is the number of bands in lane 1, b is the number of bands in lane 2 and j is the number of common bands between lanes 1 and 2. C_S values of 100% indicate that DGGE profiles are identical while C_S values of 0% indicate that the DGGE profiles are different (Waters et al., 2005).

The similarity between the DGGE profiles were determined by calculating the band similarity coefficient (S_D) (Dice: $S_D=2n_{AB}/(n_A+n_B)$, where n_A is the number of bands in line 1, n_B represents the number of bands in lane 2, and n_{AB} is the number of common bands). The similarities were represented as a dendrogram constructed using the unweighted pair group method with arithmatic means (UPGMA, BioNumerics software, Applied Maths, Austin, TX).

6.3.6 Statistical analysis

All data were analyzed using the general linear models procedure for analysis of variance (ANOVA) of SAS (1996) according to the following model: $Y_{ijk} = \mu + \delta_i + \tau_j + (\delta\tau)_{ij} + E_{ijk}$, where Y_{ijk} was the observed dependent variable (microbial diversity indexes), μ the overall mean, δ_i the house design effect, τ_j the dietary treatment effect, $(\delta\tau)_{ij}$ the interaction between house and dietary treatments effect, and E_{ijk} the random error. Before statistical analysis, all MPN data were transformed to the base-10 logarithm and replicate pens or cages of 40 birds each served as experimental units. For microbial diversity indexes pooled samples served as the experimental units for statistical analysis. Variables having a significant F-test were compared using the least-squares-means (lsmeans) function of SAS (SAS, 1996) and were considered to be significant at $P < 0.05$, unless otherwise stated. DGGE patterns were compared using Bio-Numerics Software. Cluster analysis was performed using the dice coefficient [60] for band matching with 1% position tolerance and the UPGMA method to generate the dendogram which describes the relationship between bacterial communities among dietary treatments and house designs.

6.3.7 Animal ethics

The experiments reported herein were conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry and euthanasia practices were performed with full consideration of animal welfare.

6.4 RESULTS AND DISCUSSION

Corn and triticale are considerably different in non-starch polysaccharide (NSP, dietary fiber) content, facilitating the evaluation of the effect of variation in dietary NSP levels on intestinal microbial ecology. In addition, the influence of feeding coarser rations in GIT stimulation, development, and intestinal microbial community was investigated. The finisher corn-soy diet had an average of 7.4% soluble dietary fiber (SDF) and 4.4% insoluble dietary fiber (IDF) on a dry matter basis (DM), whereas the triticale-based diets contained 10.6% SDF-DM and 7.1% IDF-DM (Table 1). Therefore, the difference in total NSP content between these two diets averaged 59.9 g/kg. Several researchers have demonstrated that increase in dietary NSP content from 2 to 40 g/kg can change animal performance, intestinal viscosity, nutrient digestibility and microbial community structure (Choct and Annison, 1992; Annison, 1993; Langhout et al., 2000; Svihus, 2001; Lan, 2004).

Dietary fiber is composed of NSP and lignin which are resistant to hydrolysis by endogenous digestive enzymes produced by animals and humans (Trowell, 1976). NSP are the primary substrates for *Lactobacillus* and *Bifidobacterium*, two important commensal bacteria found in the GIT. In general, the proliferation of microbial communities can be influenced by the source and level of dietary fiber (Högberg, 2003). For instance, a corn-soy diet contains a low amount of NSP, providing a limited amount of slowly fermentable substrate (most NSP are insoluble) for microbial growth in the hind gut of poultry (Bach Knudsen, 2001; Högberg, 2003). Conversely, a diet with high NSP content, as in wheat-based diets, provides plenty of substrate to support increased

microbial growth (Bach Knudsen et al., 1991). Thus, it was hypothesized that triticale-based diets as compared to corn-based diets would result in a greater microbial community diversity and discourage *Salmonella* colonization. Furthermore, it was expected that the beneficial effect of feeding triticale would be maximized by the use of whole grains.

The experiment was also designed to test the effect of broiler housing systems on microbial community diversity of the small intestine, and cecal *Salmonella* colonization. Avoiding contact with fecal matter by rearing broilers in the non-litter system was expected to reduce the repeated recontamination of birds with feces containing pathogens including *Salmonella*. In contrast, it was antecipated that birds on litter would be more heavily colonized by *Salmonella* which might in turn discourage the growth of some competitive bacterial species and compromise the intestinal microbial diversity.

The similarities between the DGGE profiles were first determined by cluster analysis and a dendrogram was constructed (Figure 1). As hypothesized, distinct clusters were clearly distinguished based on diet, specifically with respect to type of grain. Microbial composition within of the triticale-fed broilers were similar. Similarly, a cluster of about 60% relatedness was detected by the microbial populations derived from the corn-fed birds. Cophenetic correlation values were estimated by BioNumerics software and the values are shown in the dendrogram (Figure 1). The cophenetic correlation values were calculated based on the correlation between the dendrogram-derived similarities and the matrix similarities, and each value provides a measure of how well the dendogram represents the relationships with the input data (May, 1999;

BioNumerics, 2003). A value is also calculated for the whole dendrogram which estimates the accuracy of the cluster analysis, shown at the root of the dendrogram (BioNumerics, 2003). In this case, this value was high (89%), indicating that the dendogram did not distort the original structure in the input data (May, 1999).

The cluster analysis results were supported by the Sorenson's similarity coefficient (C_S) analysis. The Cs was used to compare average percentage similarities of DGGE banding patterns. The comparison was based on the average number of bands in common within each treatment group (Figure 2a) and between treatment groups (Figure 2b). The C_S among samples within each treatment group and between treatment groups were significantly different ($P < 0.05$, Figure 2). Statistical differences in similarities within dietary treatments showed that the similarities between samples within each dietary treatment were higher for treatments C and WT (60 and 63%, respectively) than T (31%). The C_S between treatment groups revealed that the highest value was observed among the triticale-based diets (T-WT, $C_S = 33\%$, $P = 0.019$). The lowest Cs value was observed in the comparison between corn-control diet and whole triticale (C-WT, $C_S = 13.2\%$, $P = 0.019$). Therefore, grain type and coarseness altered the intestinal community structure.

The microbial community richness (Figure 3) and diversity (Figure 4) indexes were affected by the dietary treatments, depending on housing design (diet × housing interaction $P < 0.05$). Birds raised on litter were significantly influenced by dietary treatments whereas no statistical differences were observed between treatments for broilers raised in cages ($P > 0.05$). Additionally, evenness, total band surface area and

average band surface area were not significantly affected by diet or housing design (Figures 5, 6 and 7, respectively, $P > 0.05$). Generally, the most significant contrasts observed in this study were between birds fed the whole triticale-based diets and those fed the finely ground grain (regardless of grain type) diets in the litter-reared broilers. Feeding whole triticale significantly increased the number of bands (richness, S) by 71% as compared to feeding the finely ground grain diets (9.0 vs. 5.3 bands, $P < 0.05$, Figure 3) when broilers were reared on litter. However, species evenness (E_H) was not affected by the dietary treatments (Figure 5). Therefore, as a result of increased microbial community richness, diversity (Shannon's Index, H') increased by 32% ($P = 0.04$) when broilers were reared on litter and fed the whole triticale-based diet (2.05 vs. 1.55, Figure 4c).

The cecal *Salmonella* population data (Table 2) support the intestinal microbial diversity findings. At day 42, contrast analysis showed that broilers fed whole triticale had significantly lower *Salmonella* populations than birds fed finely ground grain (3.5 vs. 4.3 log MPN/g, $P = 0.0379$). Apparently, whole grain feeding stimulated microbial community diversity, resulting in a higher level of microbial competition in the intestinal tract which perhaps discouraged *Salmonella* colonization. Similar results have been reported by Engberg and coworkers (2004) who demonstrated that whole wheat feeding reduced the number of enterococci and lactose-negative enterobacteria in the small intestine of broilers as compared to feeding pellets made with finely ground grain. Whole wheat feeding has also been shown to decrease anaerobic bacterial populations in the

gizzard (Engberg et al., 2004) and reduce intestinal colonization by *Clostridium perfringens* (Bjerrum et al., 2005).

The absence of any beneficial effect from feeding the finely ground triticale-based diet on microbial diversity indexes may be associated with low exogenous enzyme activity in the diet. The triticale-based diets were supplemented with a mixed enzyme preparation composed of endoxylanase, protease and amylase. The purpose of adding endoxylanase to a triticale-based diet was to breakdown the high level of xylan backbone present in the triticale which decreases the viscosity in the small intestine (Santos, 2006) and makes the NPS molecules more soluble and fermentable to cecal microflora (Rowe et al., 1999). Exogenous endoxylanase was supplemented in the triticale-based diets at 600 EXU per kg of feed. Recently, Santos et al. (2004) reported that supplementation of endoxylanase to wheat-based diets at 5,500 EXU/kg of feed yielded a significantly better turkey performance than did the supplementation at 2,250 EXU/kg of feed. Similarly, other investigators have also shown dose-dependent responses from supplementating diets with NSP enzymes (Hesselman et al., 1982; Petterson and Aman, 1989; Bedford and Classen, 1992). Some researchers have attribute the lack of response to the use of inappropriate enzymes for the type of grain (Friesen et al., 1992). For example, Avizyme 1502, the enzyme used in the triticale-based diets, is primarily used in poultry diets containing of corn or sorghum and soybean meal (Danisco, 2006) but generally it is not intended for use in triticale-based diets due to its low endoxylanase content. Thus, it is presumed that the concentration of added to the triticale-based diets was insufficient to completely degrade the insoluble NSP molecules present.

The triticale-based diets, which have high NSP content and large amounts of insoluble NSP, possibly provided plenty of fermentable substrate for the intestinal microflora. However, Bach Knudsen and co-workers (1991) demonstrated that these substrates are usually difficult and time-consuming to ferment. Furthermore, the passage rate through the intestinal tract is increased by feeding this kind of diet (Kass et al, 1980; Bach Knudsen, 2001). Consequently, it is likely that the presence of triticale in the diet may have selected for those microorganisms with the capacity to readily ferment NSP which may have led to a reduction in the diversity of the resident microflora. These results agree with the findings of Högberg (2003) who studied the microbial diversity of pigs fed cereal-based diets.

The microbial community diversity of birds fed whole triticale-based diets may also have been influenced by the presence of higher concentrations of endogenous glycanases. The incorporation of whole grains into pelleted broiler diets has been shown to increase the level of grain endogenous enzymes helping in the digestive processes (Jones and Taylor, 2001). Pelleting is a commercial process where small particles are combined into a larger particle by means of a mechanical process in combination with moisture, heat, and pressure (Rowe et al., 1999). The high moisture levels increase endogenous enzyme activity within the whole grains, however, when grains are ground to fine particles and subsequently pelleted, heat may inactivate these enzymes (Rowe et al., 1999; Jones and Taylor, 2001). The concentration of endogenous β-glucanase and arabinoxylanase has been shown to be significantly higher in triticale grains compared to concentrations in wheat and barley (Choct and Hughes, 2000). Therefore, it is feasible

that the presence of endogenous enzymes in the whole triticale-based diet complemented the exogenous supplemented endoxylanase resulting in the growth of different bacterial species (richness) and therefore greater microbial diversity.

The PCR-DGGE technique has been previously used to evaluate dietary effects on changes in the microbial profile of chickens (Hume et al., 2003; Lu et al., 2005) and turkeys (Waters et al., 2005; Santos, 2006). However, the method does have some limitations. Although DGGE separation of 16S variable sequences provides a convenient method to evaluate entire microbial ecosystems in a large number of samples (McCracken, 2001), multiple bands of the same bacterial species can be present in the analysis, especially when determining shifts in predominant microbial populations (Buchan et al., 2001). Buchan and co-workers (2001) demonstrated that DGGE banding patterns of environmental *E. coli* isolates obtained from different sources, including poultry, had multiple banding patterns, indicating a high degree of diversity. Nearly all bacteria contain multiple copies of the 16S rDNA gene and the copies can exhibit slight heterogeneity. It is possible that multiple bands representing the *Salmonella* species were present in the samples having elevated populations of this pathogen which would have resulted in a measure of increased richness of the samples.

The difference in *Salmonella* populations between the two housing systems was about 0.5 log or about 11,000 more cells/g in the intestinal samples taken from birds reared in the Broilermatic system. Furthermore, the richness of the flora of cage-reared broilers (regardless of dietary treatment) was equivalent to that of litter-reared broilers fed whole triticale (8.5 vs. 9.0 bands). Therefore, the increase in richness of cage-reared

broilers may be partially attributed to the higher *Salmonella* populations observed in these birds. Increasing colonization of the ceca with *Salmonella* may have also led to the colonization of the ileum by this pathogen. Hohmann and coworkers (1978) demonstrated that *Salmonella* Typhimurium can potentially colonize the small intestine and ceca of mice after oral challenge. Similarly, the observed microbial richness and diversity detected in corn fed birds raised on litter may have been caused by higher *Salmonella* populations.

In conclusion, different intestinal microbial population profiles were observed among the dietary treatments, especially among broilers reared on litter. Rearing broilers on litter and feeding whole triticale encouraged the growth of a greater variety of bacterial species and consequently a more diverse microbial community in the intestinal track. Conversely, the diversity of intestinal microflora of broilers raised in cages was not influenced by the dietary treatments. Litter-reared broilers fed finely ground grain diets, regardless of grain type, had lower microbial community diversity but higher *Salmonella* population than did those fed whole triticale. Thus, microbial community diversity seems to be influenced by the coarseness of the grain, which may be important factor impacting *Salmonella* colonization of the broiler intestine. Therefore, feeding whole high-NSP content cereals, such as triticale, may be a useful approach to control enteric pathogens in poultry with the added benefit of improved intestinal health and food safety.

6.5 CONCLUSIONS AND APPLICATIONS

1. Broilers reared in a conventional litter house and fed whole triticale had a more diverse intestinal microflora than those fed the finely ground grain, regardless of grain type.
2. Whole grain feeding presumably stimulated microbial community diversity increasing the levels of microbial competition in the intestinal tract which may have discouraged *Salmonella* colonization and reduced cecal *Salmonella* populations in broilers. Therefore, microbial community diversity seems to be an important condition impacting *Salmonella* colonization of the broiler intestine.
3. DGGE profiling is a suitable method to study microbial communities and the impact of dietary treatments and pathogen colonization.
4. Feeding whole high-NSP content cereals may be a useful approach to control enteric pathogens by improving intestinal health of poultry and benefiting food safety.

ACKNOWLEDGMENTS

This study was supported by the USDA Initiative for Future Agriculture and Food Systems (IFAFS) grant. The authors wish to thank Annette Israel, Jamie Warner, Jean de Oliveira, Ondulla Foye, Renee Plunske, Mike Mann, Robert Neely and the NCSU Poultry Educational Unit farm employees for their technical assistance. Appreciation is also extended to Southern States Feed Mill for providing and grinding the corn, to Resource Seeds Inc. for providing the triticale, and to Dr. Sophia Kathariou and Robin Siletzky for the technical assistance and equipment support with the BioNumerics software.

6.6 TABLES AND FIGURES

Table 1. Composition and nutrient content of the experimental diets containing different particle sizes of corn and triticale fed to broilers from 1 to 42 days

INGREDIENTS (%)	Starter (1 – 14 d)		Grower (15 – 28 d)		Finisher (29 – 42)	
	Corn	Triticale	Corn	Triticale	Corn	Triticale
Corn, Grain	59.90	0.00	66.78	0.00	73.27	0.00
Triticale	0.00	58.04	0.00	64.71	0.00	70.97
SBM (48% CP)	27.40	27.80	22.20	22.60	16.90	17.40
Poultry Meal (60% CP)	5.00	5.00	5.0	5.00	5.0	5.0
Poultry Oil	3.90	5.46	2.86	4.62	2.00	3.91
Limestone	1.20	1.17	1.23	1.21	1.13	1.10
Dicalcium Phosphate	1.43	1.34	0.90	0.81	0.65	0.55
Salt	0.40	0.34	0.36	0.30	0.37	0.31
Vitamin Premix[1]	0.10	0.10	0.10	0.10	0.10	0.10
Mineral Premix[2]	0.20	0.20	0.20	0.20	0.20	0.20
Choline Chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.18	0.20	0.08	0.10	0.09	0.11
L-Lysine HCl	0.04	0.00	0.04	0.00	0.04	0.00
Selenium Premix[3]	0.05	0.05	0.05	0.05	0.05	0.05
Avizyme 1502	0.00	0.10	0.00	0.10	0.00	0.10
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00
			Calculated Analysis			
ME, kcal/kg	3200	3200	3200	3200	3200	3200
Protein, %	22.0	22.0	20.0	20.0	18.0	18.0
Calcium, %	1.0	1.0	0.90	0.90	0.80	0.80
Available Phosphorus, %	0.45	0.45	0.35	0.35	0.30	0.30
Sodium, %	0.22	0.22	0.20	0.20	0.20	0.20
Lysine, %	1.20	1.20	1.05	1.06	0.90	0.90
Methionine+Cysteine, %	0.95	0.95	0.80	0.80	0.75	0.75
			Chemical Analysis[4]			
Dry Matter, %	89.75	90.96	89.30	90.02	89.45	90.33
Crude Protein, %	20.19	22.94	21.88	20.88	20.69	21.75
Gross Energy, kcal/kg	4461.26	4531.37	4484.17	4555.76	4397.17	4466.15
Fat, %	7.10	8.07	6.82	7.66	6.04	6.28
Ash, %	5.68	5.59	4.75	5.53	5.66	4.27
Fiber Total, %	12.87	18.40	22.54	23.09	11.79	17.71
Insoluble, %	5.17	7.43	10.00	9.80	4.44	7.07
Soluble, %	7.70	10.97	12.54	13.30	7.36	10.64

[1] The vitamin premix supplied the following per kilogram of feed: vitamin A, 26,400 IU; cholecalciferol, 8,000 IU; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4 mg; pyridoxine, 15.8 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 8 mg; biotin, 0.506 mg; vitamin B12, 0.08 mg; ethoxyquin, 200 mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate.

Table 1. Continued

[2] The mineral premix supplied the following per kilogram of feed: 120 mg Zn as $ZnSO_4 \cdot H_2O$; 120 mg MN as $MnSO_4 \cdot H_2O$; 80 mg Fe as $FeSO_4 \cdot H_2O$; 10 mg Cu as $CuSO_4$; 2.5 mg I as $Ca(IO_3)_2$; 1.0 mg Co as $CoSO_4$.

[3] Selenium premix provided 0.3 ppm Se from sodium selenate.

[4] Chemical Analysis: Crude protein determined using Kjeldahl automatic analyzer (Kjeltec Auto 1030 Analyser, Tecator, Sweden). Gross energy determined using bomb calorimeter (IKA Calorimeter System C5000 control, IKA® Werke Labortechnik, Staufen, Germany). Fat determined by ether extract (Labconco Corporation, Kansas City, MO) method. Ash determined by muffle oven (Thermolyne, Sybron Corporation, Dubuque, IA) method. Dietary fiber analysis was conducted by Intertek Agri Services Food Agricultural Testing Laboratory (St. Rose, LA) using the AOAC International standard method for soluble, insoluble and total dietary fiber analysis.

Table 2. Cecal *Salmonella* populations of broilers fed corn- or triticale-SBM diets and raised in a conventional litter floored house or in the Broilermatic System at 42 days of age

Housing Design	**log MPN/g**
Litter Floor	3.75
Broilermatic	4.22
P-value	0.1375
SEM (6)[1]	0.1955
Dietary Treatments	**log MPN/g**
C[2]	4.39
T[3]	4.10
WT[4]	3.50
P-value	0.0825
SEM (6)	0.2395
Source of Variation	**(P-value)**
House × Diet	0.2354
Corn × Triticale	0.0835
Ground × Coarse	0.0379

[1] SEM (6): standard error of the mean with 6 degrees of freedom.
[2] C: finely ground corn.
[3] T: finely ground triticale.
[4] WT: whole triticale.

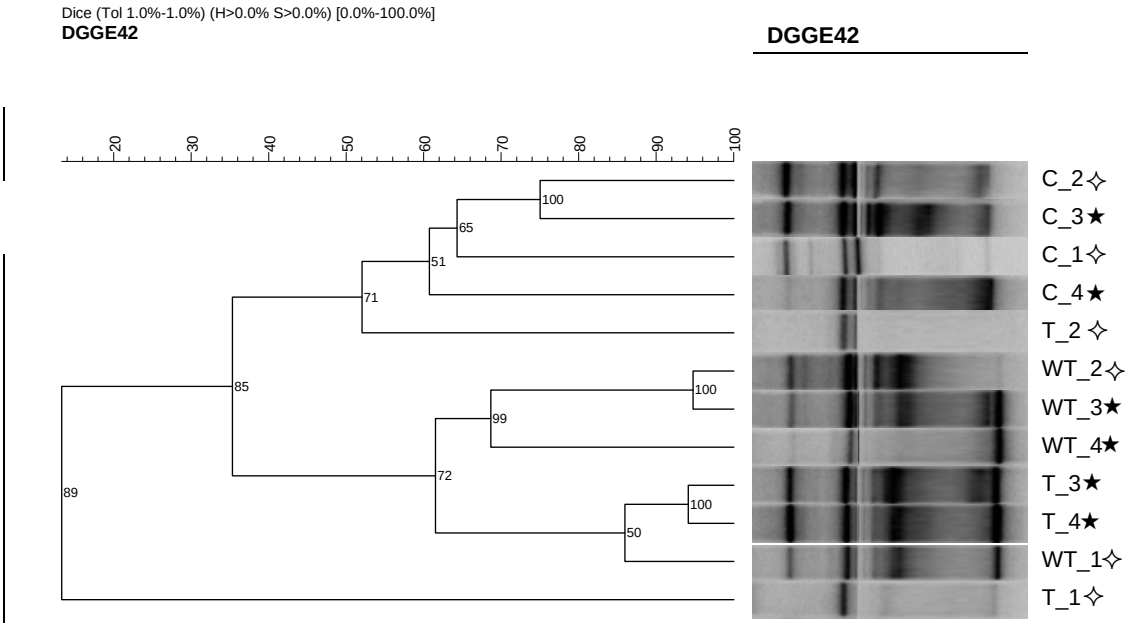


Figure 1. Cluster analysis of the DGGE profiles of microbial populations present in the ileum contents of 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets and reared in a litter floor house (✧) or in the Broilermatic non-litter cage system (★). The dendrogram was band-based and was constructed using the unweighted pair group method with arithmatic means (UPGMA) using the BioNumerics software (Applied Maths, Austin, TX). Cophenetic correlation coefficients are represented on the root of each cluster in the dendogram (relatedness tree). This parameter is used to express the consistence of a cluster, and represents the correlation between distance values calculated during tree building and the observed distance (BioNumerics, 2003). Clusters (groups) were determined by sequential comparison of band patterns and the results (relative similaties) are represented in the dendrogram. Similarity between samples is indicated by the percentage coefficient bar located above the dendrogram. Numbers accompanying the dietary treatments (C, T and WT) represent the replicate samples (4 replicates/treament). Each sample represents a pool of ileum contents of 4 birds. Distances are measured in arbitrary units.

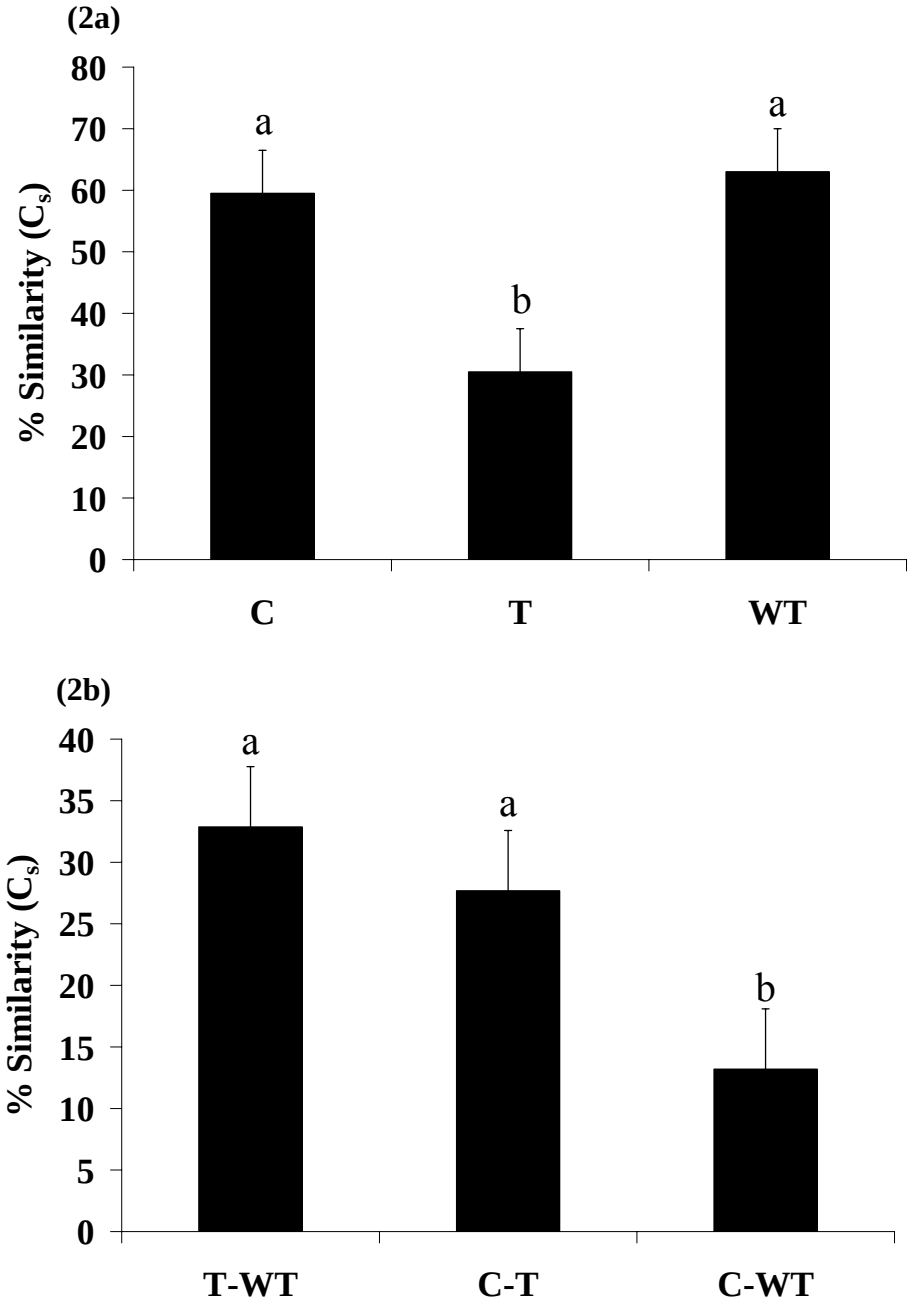
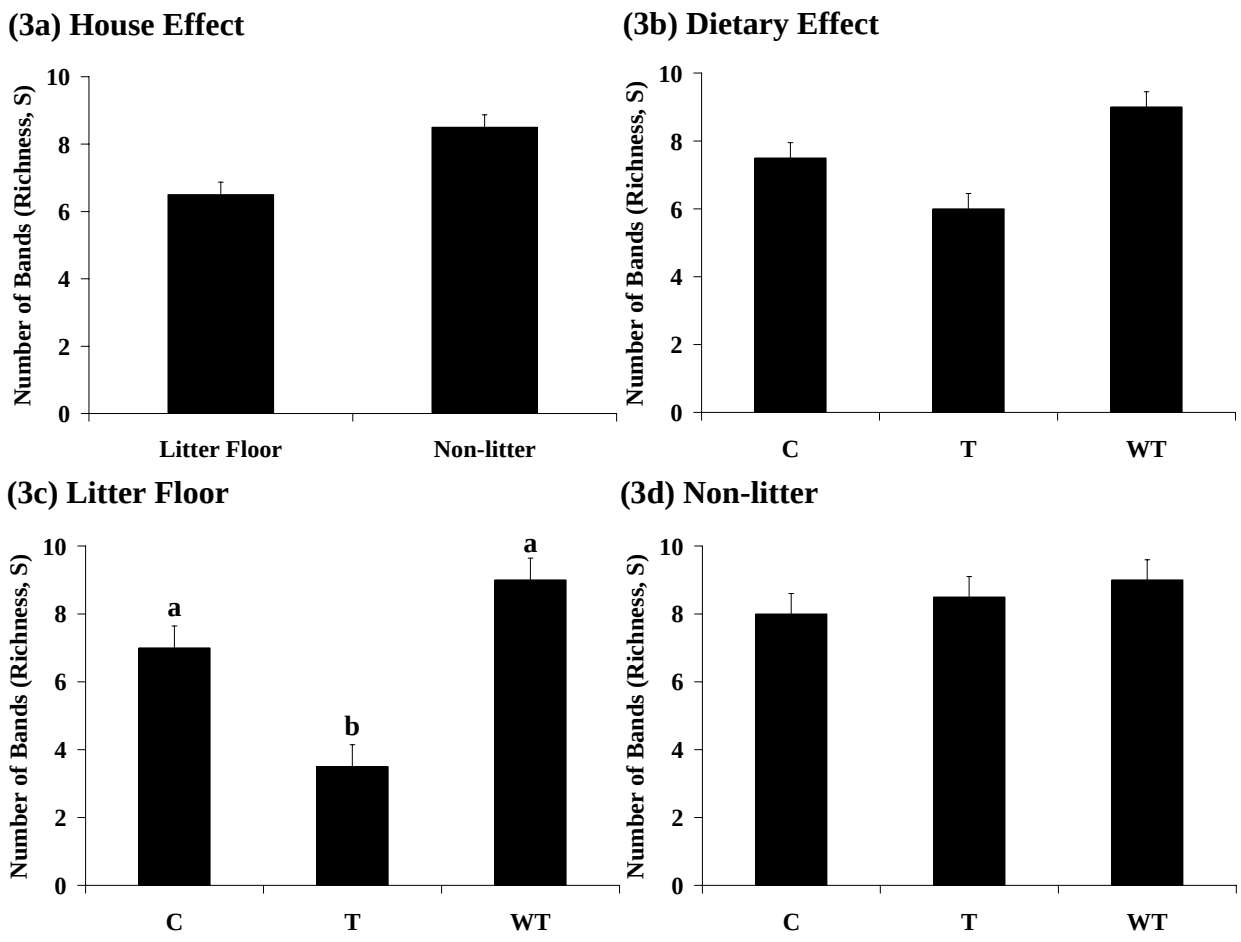
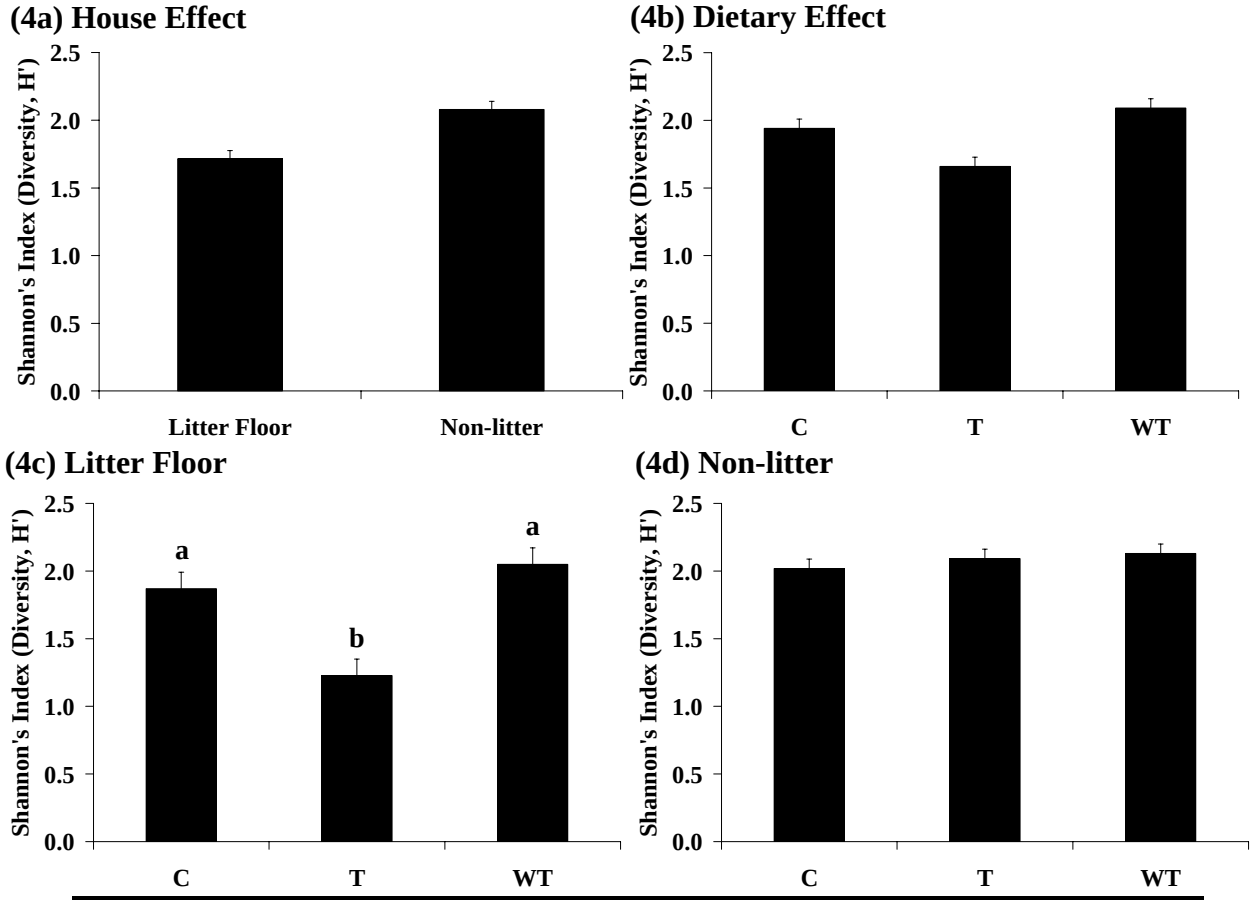


Figure 2. Percentage of similarities of DGGE banding patterns from bacterial DNA of the ileum contents of 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Sorenson's similarity index (% similarities, y-axis) is based on the average number of bands in common within each dietary treatment (2a) and between dietary treatments (2b) across housing designs. Calculations were based on the formula: $C_s = [2j/(a+b)]\times100$, where a is the number of bands in lane 1, b is the number of bands in lane 2 and j is the number of common bands between bands 1 and 2. Values represent means from each group of comparison (dietary treatments, x-axis). Values sharing different superscript letters within the charts are statistically different ($P < 0.05$).



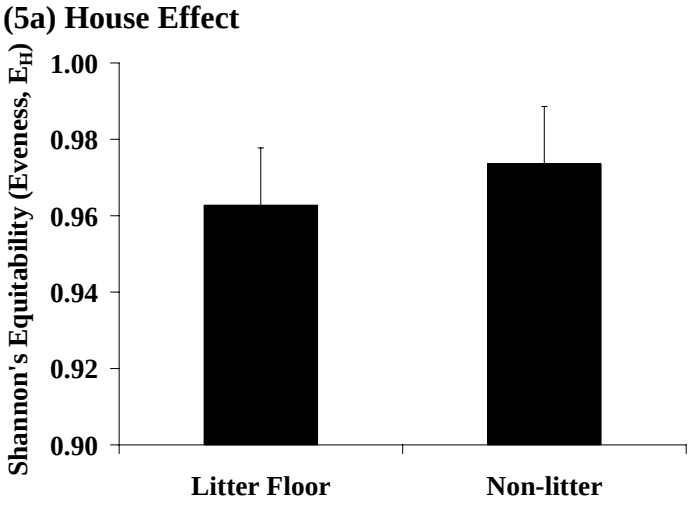
Statistical Analysis		
Source of Variation	**P-Value**	
House Effect	0.0090	
Diet Effect	0.0103	
Diet × House	0.0182	
Corn × Triticale	1.0000	
Ground × Whole	0.0069	
By Housing Design	***Litter***	***Non-litter***
Diet effect	0.0204	0.6037
Corn × Triticale	0.4128	0.4128
Ground × Whole	0.0178	0.4128

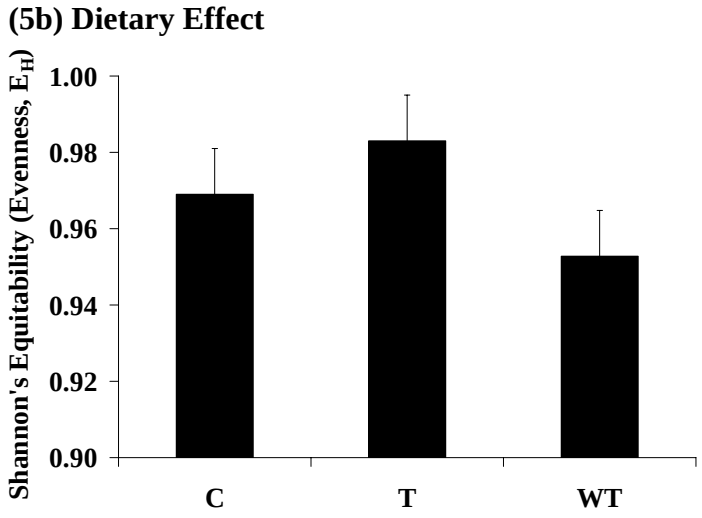
Figure 3. Species richness of microbial populations present in the ileum contents of 42 d broilers fed finely corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Richness (S-index, y-axis) was calculated based on the average number of bands of each sample for the corresponding housing design across dietary treatments (3a), dietary treatment across housing design (3b) and within housing design (3c and 3d). Treatment groups sharing different superscript letters are statistically different ($P < 0.05$).



Statistical Analysis		
Source of Variation	**P-Value**	
Diet Effect	0.0123	
House Effect	0.0039	
Diet × House	0.0127	
Corn × Triticale	0.4410	
Ground × Whole	0.0146	
By Housing Design	***Litter***	***Non-litter***
Diet effect	0.0344	0.5542
Corn × Triticale	0.2167	0.3401
Ground × Whole	0.0435	0.4260

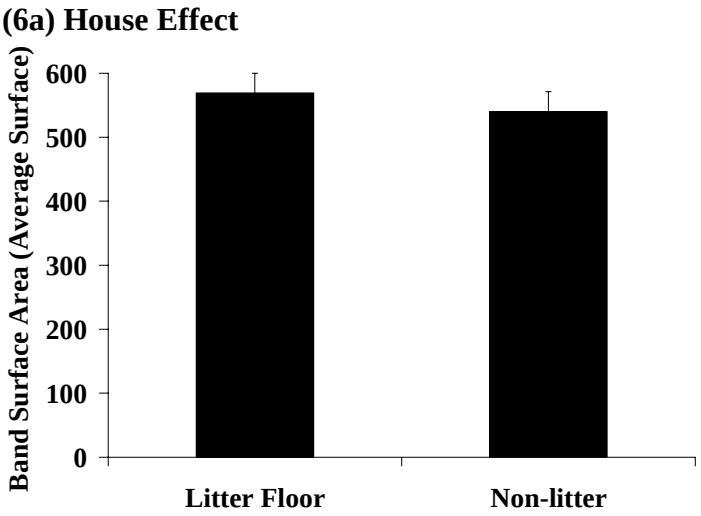
Figure 4. Diversity of microbial populations present in the ileum contents of 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Diversity or Shannon's index (H' index, y-axis) was calculated based on the formula: $H' = -\Sigma p_i \ln p_i$, where p_i is the proportion of individuals in the population belonging to the i^{th} species, which corresponds to the proportional abundance of band i. Values represent the mean of each housing design across dietary treatments (4a), dietary treatments across housing design (4b) and within housing design (4c and 4d). Treatment groups sharing different superscript letters are statistically different ($P < 0.05$).

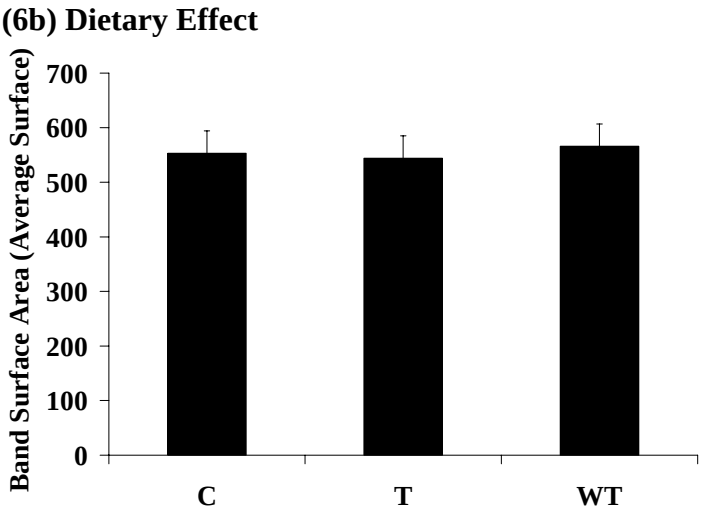




Statistical Analysis	
Source of Variation	**P-Value**
Diet Effect	0.5300
House Effect	0.6128
Diet × House	0.6170
Corn × Triticale	0.9632
Ground × Whole	0.3329

Figure 5. Species evenness of microbial populations present in the ileum contents of 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Evenness was calculated using the Shannon's equitability index (E_H index, y-axis) based on the formula: $E_H = H'/\ln S$, where S is the total number of species in the community (total number of bands, richness) and H' (diversity index) can be calculated by: $H' = -\Sigma p_i \ln p_i$, where p_i is the proportion of individuals belonging to the i^{th} species (proportional abundance of band i). Values represent the mean of each housing design across dietary treatments (5a) and dietary treatment across housing design (5b).



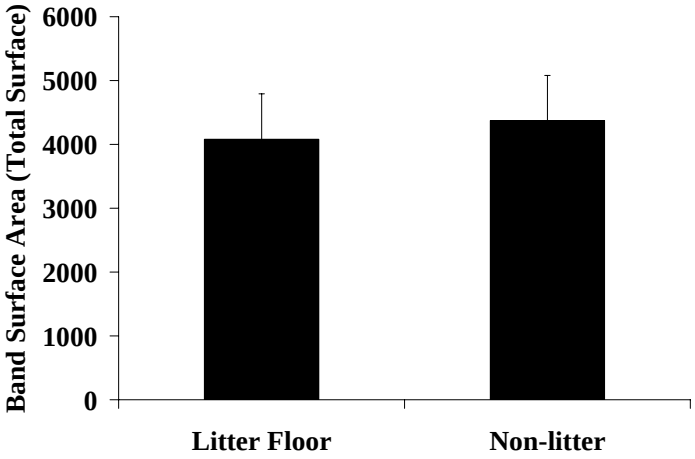


Statistical Analysis

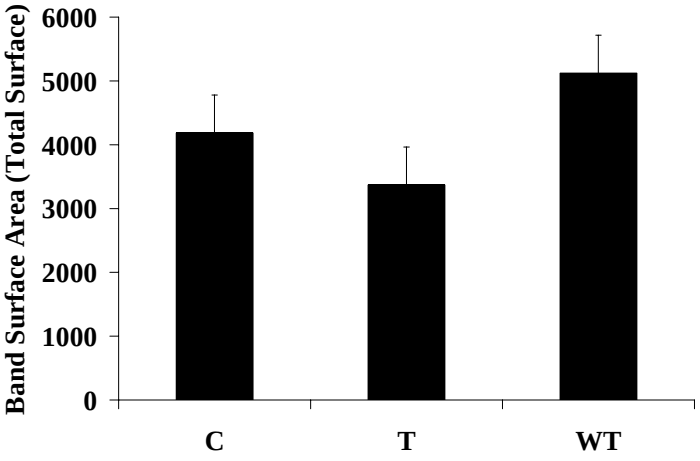
Source of Variation	P-Value
Diet Effect	0.5300
House Effect	0.6128
Diet × House	0.6170
Corn × Triticale	0.9332
Ground × Whole	0.3329

Figure 6. Average surface area plots of denaturing gradient gel electrophoresis (DGGE) bands from bacterial DNA of the ileum contents of 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Band surface area corresponds to measurements of the optical density of each band. The optical density was measured based on the band intensity and migration distance in the gel. The y-axis represents the optical density and the x-axis represents the dietary treatments. Average surface area was determined by averaging the surface area of each band within each lane of each housing design across dietary treatments (6a), and within each dietary treatment across housing designs (6b).

(7a) House Effect



(7b) Dietary Effect



Statistical Analysis

Source of Variation	**P-Value**
Diet Effect	0.6269
House Effect	0.7814
Diet × House	0.7022
Corn × Triticale	0.3773
Ground × Whole	0.8482

Figure 7. Total band surface area plots of denaturing gradient gel electrophoresis (DGGE) bands from bacterial DNA of the ileum contents 42 d broilers fed finely ground corn (C), finely ground or whole triticale (T and WT, respectively) based diets. Band surface area corresponds to measurements of the optical density of each band. The optical density was measured based on the band intensity and migration distance in the gel. The y-axis represents the optical density and the x-axis represents the dietary treatments. Total surface area was determined by summing the surface area of all bands within each lane of each housing design across dietary treatments (7a), and within each dietary treatment across housing designs (7b).

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CHAPTER 7

SUMMARY

Significant improvements in the performance of commercially reared broilers and turkeys have been made over the last two decades. These improvements include increases in live weight, better feed efficiency, and reductions in mortality. These gains in production may be the direct result of genetic selection for faster growth, improved nutrition and flock health, and better management practices. Food safety improvements were also seen on the post-harvest sector of the poultry industry, especially after the implementation of the Hazard Analysis Critical Control Points (HACCP) program in 1996. The industry has made significant advancements in the reduction in incidence of poultry carcass contamination with *Salmonella*. However, salmonellosis, especially of foodborne origin, is still one of the most common causes of gastro-intestinal infections in the United States and many countries around the world. Moreover, many poultry farms still face the problem of endemic *Salmonella* colonization of their flocks. In order to prevent or reduce *Salmonella* populations from these environments, it is necessary to prevent its introduction or re-introduction onto the farm. Because carcass contamination with *Salmonella* has been linked to colonization of live birds on the farm, further reductions in post-process contamination will require on-farm interventions. Most *Salmonella* investigations previously conducted at the poultry production level were based on *Salmonella* prevalence, which neglected the importance of populations of the organism, when present. Furthermore, it is very important to identify significant sources of *Salmonella* contamination at the pre-harvest level so that corrective actions to reduce or eliminate these contamination sources can be determined, which will ultimately result in a reduction in pathogen load on and in the bird.

To address the challenges that the poultry industry is facing regarding the control of *Salmonella*, it was hypothesized that the application of alternative management practices on

the farm such as housing design and dietary manipulations including altering dietary grain particle size or fiber composition can alter the intestinal microbial community and reduce *Salmonella* colonization of poultry. To test this hypothesis five experiments were conducted and their results are summarized below.

The objectives were to first assess *Salmonella* populations present on brooder and grow-out North Carolina turkey farms (litter and fecal samples) using a quantitative procedure (most probable number–MPN, **first experiment, chapter 2**) followed by an investigation of the diversity of *Salmonella* serotypes and genotypes present on these farms using serotyping, genotyping by pulsed-field gel electrophoresis, and antibiotic resistance-susceptibility analyses (**second experiment, chapter 3**). Additionally, alternative on-farm pathogen intervention strategies including feeding whole grains such as corn and increasing the insoluble fiber content of the diet (**third experiment, chapter 4**) were evaluated. The use of alternative grains such as triticale (finely ground and whole) in contrast to corn and an alternative non-litter (Broilermatic Cage System) housing design (**forth experiment, chapter 5**) were also tested. Lastly, the influence of feeding triticale (finely ground and whole) in comparison to corn and rearing broilers in a non-litter cage system versus a conventional litter floored house on ileal microbial diversity were evaluated and compared to changes in *Salmonella* cecal colonization (**fifth experiment, chapter 6**).

Salmonella litter populations of commercial turkey farms averaged 2 logs higher in 3-wk poult samples compared to those from 19-wk birds (4.1 vs. 2.1 log MPN/g). *Salmonella* was present in 70% of the fecal and 79% of the litter samples taken during the brooder and grow-out phases of commercial turkey production. Season did not influence *Salmonella* litter or fecal populations, yielding an overall average population of 3.0 log MPN/g of sample.

These results provided evidence that *Salmonella* are present at high populations during turkey production even when bacterial cells are exposed to apparently adverse environments. These findings suggest that intervention strategies should aggressively target critical points such as better litter management on brooder farms and better control of the factors that may increase the bird's susceptibility to pathogen colonization, including temperature-induced stresses.

The diversity of *Salmonella* serotypes isolated from commercial turkey farms also varied significantly depending on turkey age. Serotypes Kentucky, Heidelberg, Hadar and 8,(20):nonmotile were only isolated from samples obtained from 19-week old turkeys, whereas Senftenberg and Muenster were only isolated from 3-week old poults. Antibiotic resistance patterns of *Salmonella* serotypes were significantly influenced by turkey age. Isolates from turkey poults (3 wk) were resistant to more than 4 antibiotics whereas those from older turkeys (19 wk) were resistant to 3 or less antibiotics. Given the fraction of isolates that were analyzed, no farm or seasonal effects were observed on serotype distribution or antibiotic resistance patterns. A total of 12 PFGE clusters of 65% or greater relatedness were observed across the *Salmonella* isolates. Turkey age was significantly related to the distribution of genotypes between clusters. The combined analysis of genotypes and phenotypes provides a more complete understanding of *Salmonella* populations during turkey production and confirms the significant influence of turkey age on *Salmonella* serotype distribution.

The use of coarse ground corn and increased insoluble fiber (wood fiber) content in corn-based turkey diets as opposed to using finely ground corn improved the feed conversion ratio (FCR) during the critical 7 d post-hatch period and did not adversely impact bird body

weights. Additionally, the positive effect of dietary supplementation of wood shavings continued through 14 d as the poults consumed less feed than the other two treatments without adverse effects on body weight. Supplementation of the diet with wood shavings decreased the relative small intestine and jejunum weights, which is likely related to the improved feed conversion ratio detected among the birds receiving wood shavings as less energy is required to maintain the gastrointestinal tract. Diets formulated with coarse ground corn or wood shavings yielded turkeys with increased relative gizzard weights and possibly improved digestion and absorption of nutrients as the gizzard is an important organ in the digestion process and greatly influences intestinal motility. Particle size and structure of feed components had no effect on *Salmonella* colonization.

To examine the impact of housing design, the use of grains other than corn and the addition of whole grains to the diets (whole triticale and coarsely ground corn), broilers were reared on four different diets to market age (42 d) and *Salmonella* colonization was measured. The diets were finely and coarsely ground corn and finely ground and whole triticale. Whole grain supplementation decreased *Salmonella* cecal populations whereas rearing broilers on litter floor as opposed to the non-litter Broilermatic cage system resulted in significant reductions in *Salmonella* colonization. The results also showed that dietary NSP content, housing design, grain type and particle size influenced the bird's intestinal size and histomorphometry as it adapts to changes in nutrient digestibility. Furthermore, dietary inclusion of coarse grain particles or a high dietary fiber content grain, such as triticale, discouraged *Salmonella* colonization of broiler ceca. Rearing broilers in conventional litter-based housing was superior to a non-litter housing system in terms of growth performance and resistance to colonization following *Salmonella* challenge.

The effects of grain, particle size, and type of housing system on intestinal microbial diversity of broilers were also investigated. The combination of high NSP-content and increased grain particle size such as feeding whole triticale increased microbial community diversity and discouraged *Salmonella* colonization as compared to finely ground grain diets. Furthermore, the beneficial effect of feeding triticale was enhanced by the use of whole grain. The experiment was also designed to test the effect of housing systems on broiler microbial community diversity and *Salmonella* colonization of the small intestine. Broilers reared in a conventional litter floor house and fed whole triticale had a more diverse intestinal microflora than cage-reared birds (non-litter system). Moreover, feeding whole triticale presumably encouraged the proliferation of beneficial bacterial populations which may have competitively excluded *Salmonella* in the ceca of broilers. Therefore, DGGE profiling may be a suitable method to study microbial communities and the impact of dietary treatments on pathogen colonization.

In conclusion, this dissertation presents evidence that variable *Salmonella* populations, serotypes and genotypes are present across commercial turkey farms. In addition, various degrees of resistance to antibiotics were observed among the *Salmonella* isolates. A daily intake of fiber seemed to be beneficial for turkeys and broilers as previously shown for humans and other animals. Turkeys supplemented with 4% (w/w) ground wood shavings had better feed conversion ratios than those that were not supplemented. Additionally, feeding triticale especially in the whole grain form influenced enteric health, intestinal anatomy, intestinal development and intestinal function, particularly when broilers were challenged with enteric pathogens such as *Salmonella*. Supplementing diets with exogenous NSP-degrading enzymes reduced the adverse effects of dietary NSP on nutrient

digestibility and likely liberated different types of non-starch oligosaccharides that served as substrates for a more diverse microflora. Maintaining a stable ecosystem that prevents the colonization of unfavorable microbial communities can apparently benefit growth performance of turkeys and broilers. Therefore, diet formulation can have a significant influence on *Salmonella* colonization of poultry. Moreover, diet composition and grain coarseness as well as housing design can greatly influence the diversity of the commensal intestinal microflora which can potentially help in the control of *Salmonella* colonization in broiler intestines. Furthermore, dietary inclusion of whole high-NSP content cereals and fiber-degrading enzymes can be used as a method to reduce the risk of *Salmonella* contamination of poultry intended for human consumption.